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THE UNIVERSITY OF ALBERTA

UNIDIRECTIONAL CONVERSION IN NEUROSPORA MITOCHONDRIAL DNA

by

ALEXISANN HAWSE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

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EDMONTON, ALBERTA

FALL 1988

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled UNIDIRECTIONAL CONVERSION IN NEUROSPORA MITOCHONDRIAL DNA submitted by ALEXISANN HAWSE in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

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This thesis is dedicated to my parents, Alex and Bev and to the memory of my dear friend, Paul Fortin, for believing in me - and my dreams.

ABSTRACT

In heterokaryons forced between two strains of *Neurospora crassa* carrying either the type I or type II polymorphic forms of mtDNA, it was previously observed that within the mtDNA of the heterokaryons, EcoR1-5 fragments were rapidly and completely converted to the type II polymorphic form and 70% of the heterokaryons were converted to the type I polymorphic form of the EcoR1-9 fragment (Mannella and Lambowitz, 1978; Mannella and Lambowitz, 1979). In a separate study, in which different strains were used, conversion of the EcoR1-9 fragment was 70% to the type II form (Lemire and Nargang, 1986).

Sequence analysis performed on the type II form of EcoR1-5 by Burger and Werner (1985) revealed the presence of a large URF containing intron within the URF1 gene. The sequence analysis performed in the present study revealed that the smaller, type I form of EcoR1-5 arises from the precise excision of the URF containing intron. In this study the DNA sequence of the type I and type II EcoR1-9 fragments were determined entirely. The only difference found between the two polymorphic forms was the presence of an extra copy of a 78 base pair repeat. This repeat is found three times in the type I form of EcoR1-9 and twice in the type II form.

Although the predicted amino acid sequence of the URF within the URF1 gene contains no detectable homology to the omega protein of yeast, the conversion of the EcoR1-5 fragment may occur in a fashion analogous to that observed for the omega intron (Colleaux *et al.*, 1986) The results of previous workers suggested that the direction of conversion of the EcoR1-9 fragment depends on the phenotype of the heterokaryon component strains. Heterokaryons formed between [*poky*] (type IIa mtDNA) and wildtype strains containing type I mtDNA resolved 70% of the heterokaryons to the type I polymorphic form of EcoR1-9 (Mannella and Lambowitz, 1979) whereas heterokaryons formed between [*mi-3*] (type I mtDNA) and wildtype strains containing type II mtDNA) appeared to resolve 70% of the heterokaryons to the type II polymorphic form of EcoR1-9 (Lemire and Nargang, 1986). The present study confirms the 70% conversion to the type II mtDNA form of the EcoR1-9 fragment in [*mi-3*]/wildtype heterokaryons.

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ABREVIATIONS

bp	base pairs
dH_2O	distilled water
IPTG	Isopropylthio-ß-D-galacotside
mtDNA	Mitochondrial DNA
ORF	Open Reading Frame
RPM	revolutions per minute
URF	Unidentified Reading Frame
X-gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactoside

INTRODUCTION

The Organism

Neurospora crassa is a member of the fungal class Ascomycetes. Its value as a laboratory organism arises from the fact that it has a short life cycle, easily identifiable characters, and because its different cellular fractions are easily isolated. *Neurospora* is a heteromorph. During vegetative growth it is composed of multinucleate, branched filaments called hyphae. Growth proceeds by hyphal tip extension and by development of branches behind the tips. This hyphal system is referred to as mycelium. The hyphae are segmented by incomplete cross walls or septa which contain central pores of about 0.5μ in diameter. These pores allow cytoplasm to flow along the hypha, carrying nuclei, mitochondria, and other cytoplasmic components for some distance, usually in the direction of growth. The nuclei divide most rapidly near the hyphal tips (Davis and de Serres, 1970).

There are two asexual spore forms. The smaller, called microconidia, are formed late in growth and are uninucleate. The larger, called macroconidia, are multinucleate (average ca. 2.5 nuclei per spore). Because they are more viable than microconidia the macroconidia usually act as the male fertilizing parent when forming sexual crosses. The products of the sexual cycle in *Neurospora* are referred to as ascospores. The sexual cycle requires strains of opposite mating types and is stimulated by conditions non-conducive to vegetative growth, such as nitrogen starvation (Davis and de Serres, 1970). In sexual crosses, mitochondria are maternally inherited and there is no interaction between the mitochondria of the male and female strains.

Heterokaryons allow one to study the interaction of different extranuclear characteristics found in the same cytoplasm since when heterokaryons are formed, the nuclei and mitochondria from different strains are brought together in the same cytoplasm (Davis and de Serres 1970). Heterokaryons may be forced between strains with compatible nuclear genomes by superimposing conidia from strains with different auxotrophic markers onto minimal media (Davis and de Serres 1970).

Neurospora extranuclear mutants have been placed into four groups based on phenotypic and genotypic relationships (Bertrand *et al.*, 1976). Group I mutants are deficient in cytochrome aa_3 and b. These mutants are defective in mitochondrial ribosome assembly (Bertrand *et al.*, 1976). This assembly defect has been related to a deletion in the 5' end of the mitochondrial small rRNA (Akins and Lambowitz,

1979). Of the seven known group I mutants, six of these were examined and found to carry the deletion and it has been suggested that all members of this group are actually reisolates of the original [*poky*] mutant (Akins and Lambowitz, 1979). The deficiency of cytochrome aa3 and b in the mutants is thus explained by an inability to translate the mitochondrially encoded subunits of these cytochromes.

The two mutants [*mi-3*] and [*exn-5*] make up the second group of mutants. These mutants are noted for their initial lag phase and are deficient in cytochrome aa3. The [*mi-3*] mutant has been found to be due to a point mutation in the gene encoding subunit 1 of cytochrome c oxidase (Lemire and Nargang, 1986). The [*exn-5*] mutant has been reported to be the result of a mutation in the gene encoding subunit two of cytochrome c oxidase (Lemire, 1988)

The third group of mutants have a characteristic start-stop growth phenotype and thus are known as "stopper" mutants. Lesions affecting mitochondrial genes required for protein synthesis leads to their characteristic deficiency in cytochromes aa3 and b because the subunits of these cytochromes that are encoded in the mitochondria cannot be translated (Bertrand *et al.*, 1976) Gross *et al.* (1984) and de Vries *et al.* (1986) noted that stopper mutants have two circular pieces of DNA present during the stopped phase which arise as the result of recombination between repeated sequences in the mitochondrial genome. Thus it appears that the lesions affecting protein synthesis in these stopper mutants are the result of deletions formed by recombination between repeats (Gross *et al.*, 1984; de Vries *et al.*, 1986).

Cni-3 has a normal cytochrome system, but this lone group IV mutant constitutively induces the mitochondrial cyanide-insensitive alternate oxidase (Rosenberg *et al.*, 1976).

Suppressivity

Suppressivity in *Neurospora* occurs when the mutant mitochondrial phenotype of one strain predominates over the phenotype of another strain in a heterokaryon formed between the two different strains. Early studies on the cytoplasmic mutants [*abn-1*] and [*abn-2*] of *Neurospora crassa* showed that mitochondria are capable of transmitting cytoplasmic characters (Diacumakos *et al.*, 1965; Garnjobst *et al.*, 1965). The two mutants differ from wildtype in that they have a characteristic slow growth rate and are abnormal in appearance. [*abn-1*] was isolated from an inositolless strain and [*abn-2*] was isolated from a wildtype strain. Garnjobst and colleagues (1965) found [*abn-1*] and [*abn-2*] strains of *Neurospora*

did not transmit their [*abn*] characterstics when used as the male parent in sexual crosses, but the abnormal mutant characteristics were always transmitted when heterokaryons were formed with wildtype strains. These results suggested that a cytoplasmic factor was responsible for the [*abn*] phenotype. Garnjobst *et al.* (1965) confirmed this when they found that wildtype cultures that had been microinjected with cytoplasm from abn cultures became phenotypically [*abn*]. Diacumakos *et al.*, (1965) extended this study by injecting purified mitochondrial fractions from an inositolless;[*abn-1*] strain into single hyphal compartments of normal strains. Mitochondrial fractions from wildtype strains produced no effect whereas the [*abn1*] injected mitochondrial fractions caused the normal strains to aquire the characteristics of the [*abn-1*] strain without transmission of the nuclear encoded inositolless trait (Diacumakos *et al.*, 1965).

Studies on extranuclear mutants, such as the [*poky]* mutant, showed that the mitochondrial phenotype of the [*poky*](or [*mi-1*]) extranuclear mutant could also predominate and thus are also considered supressive over the wildtype phenotype in heterokaryons forced between the two. (Pittenger, 1956; Mannella and Lambowitz, 1978; Mannella and Lambowitz, 1979) Mannella and Lambowitz (1978) utilized restriction enzyme analysis (described below) and naturally occurring polymorphic forms of mtDNA to investigate suppressivity in heterokaryons forced between [*poky*] and wildtype strains of *Neurospora crassa* . It was concluded that the [*poky*] phenotype predominates in such heterokaryons since after two to eight conidial passes, 90% of the heterokaryons displayed the [poky] characteristics of slow growth rate and an altered ratio of 19S to 25S mitochondrial rRNAs . (Mannella and Lambowitz, 1978; Mannella and Lambowitz, 1979)

Gowdridge (1956) reported results opposite to Pittenger (1956) and Mannella and Lambowitz (1979); the wildtype phenotype predominated in her heterokaryon studies. In Gowdridge's studies heterokaryons were formed on minimal slants, transferred to plates and the hyphal tips used to start another culture. The "hyphal tip" cultures were analyzed with respect to growth rates and cytochrome content to establish the phenotype of the heterokaryon. Since Gowdridge's analysis covered only two culture passes, the different results observed by Gowdridge (1956) for the [*poky*] / wildtype heterokaryons are probably due to the insufficient number of conidial passes in her studies. Two to three generations may not have provided enough of an opportunity for the [*poky]* mitochondria to take over.

Gowdridge also examined heterokaryons formed between [*mi-3*] and wildtype and found that the [*mi-3*] phenotype predominated in about 50% of the cases. Lemire and Nargang (1986) performed eight conidial transfers as well as passage through a race tube in order to guarantee a homokaryotic culture. They found that only 30% of the heterokaryons acquired the [*mi-3]* phenotype.

Gowdridge also formed heterokaryons between [*mi-3*] and [*poky]*. In these heterokaryons, the [*mi-3*] phenotype appeared to predominate over [*poky*]. (Gowdridge, 1956) These results are not reliable as it was difficult to distinguish between the [*poky*] and [*mi-3*] phenotypes by the techniques employed in this study.

The extranuclear "stopper" mutants of *Neurospora* also appear to be suppressive.(Gross *et al.*, 1984) The suppressivity of "stopper" mutants may be related to the mitochondrial deletions found to be associated with the stopper phenotype (de Vries *et al.*, 1986, Gross *et al.*, 1987).

In yeast, suppressivity has been observed with the petite mutants. When crosses are made between petite mutant strains and wildtype strains a non-mendalian segregation of the mutation is observed in the diploids formed. The proportion of petite colonies to wildtype in the offspring depends on the particular petite strain used. The "petite colonie" mutation of *Saccharomyces cerevisiaee* is characterized by an irreversible loss of respiration and by an extremely high spontaneous occurrence. (de Zamaroczy *et al.*, 1981) These petite colonies are due to mutations in the mtDNA, defined as rho$^-$ mutants. Rho$^-$(ρ^-) cells are non-reverting pleiotropic mutations with extensive deletions in the mtDNA (or complete loss of the mitochondria, designated ρ^o mutants). (Dujon, 1981) The mitochondrial genomes of the suppressive ρ^- (petite) mutants are made up of tandem repetitions of a DNA sequence excised from the wildtype parental genome and carries at least one of the seven known ori sequences. (de Zamaroczy *et al.*, 1981) The suppressive petite mitochondrial genome is preferentially transmitted to the progeny of crosses between petite and wildtype cells. The level of suppressivity of a petite mutant appears to depend on the relative replication efficiencies of petite versus wildtype genomes. The multiple copies of ori sequences, due to the tandem amplification of the petite repeat unit, allow the mutants to replicate more efficiently than the wildtype genome. The different levels of supressivity may be due to the size of the repeat unit since the shorter the repeat unit the higher the number of ori sequences in the mutants mitochondrial genome.

Thus the greater the density of ori sequences in the petite's mitochondrial genome, the greater the replication efficiency which results in its enhanced suppressivity. (de Zamaroczy *et al.*, 1981) In support of these findings Bernardi (1983) reported that partial or total deletions and rearrangements of the ori sequence(s) depresses the suppressivity of the corresponding petites.

Although the phenotypes of mit$^-$ and ρ^- mutants appear to be similar (formation of small colonies on glucose substrates and inability to grow on non-fermentable substrates), mit$^-$ mutants retain their ability for mitochondrial protein synthesis (Tzagoloff, 1982). Because mit$^-$ muations in yeast mtDNA are due to point mutations that affect one of three enzymes (Tzagoloff, 1982) they would not be induced to become suppressive by amplification of any part of its genome. The same can be said for syn$^-$ mutants which are similar to ρ^- strains in that they are deficient in the respiratory and ATPase complexes but genotypically the syn$^-$ mutants are due to specific point mutations that affect mitochondrial protein synthesis as apposed to the large mtDNA deletions found in ρ^- strains (Naysmith, 1982; Tzagoloff, 1982).

Neurospora is an obligate aerobe and requires an intact mitochondrial genome. Therefore, suppressivity in *Neurospora* must differ from the situation in yeast because gross structural alterations associated with *Neurospora* mtDNA would be lethal. Thus, large deletions of the mitochondrial genome are not associated with suppressivity in *Neurospora* . The *Neurospora* mtDNA origin of replication has not yet been defined and there is no known mechanism for suppressivity in this organism although segregation of mitochondria may play a major role (Birky *et al.*, 1982; Backer and Birky, 1985). Segregation of mitochondria may also play a role in the mechanism of take over by a new mutation in the mtDNA population.

Eukaryotic cells contain many mitochondria. Each mitochondrion contains multiple copies of the mitochondrial genome and therefore, many copies of each mitochondrial gene exist in the cell. (Birky *et al.*, 1982). The frequency of an allele within the mitochondrial population of genes will depend on the input frequency, random drift and random segregation of the mitochondria into daughter cells (Backer and Birky, 1985). Backer and Birky (1985) found that in yeast, when there is no selection, random drift and random partitioning during cell division are important in determining the fate of a new mutation. Therefore, one would rarely see a new mutation fixed in the population. This may explain the small

number of extranuclear mutants observed in *Neurospora* since, with no selective pressure, a spontaneous mitochondrial mutation would quickly be lost. In the case of equal input of two alleles and no selective pressure, one would expect an equal representation of both alleles in the population. There must be some selective advantage that exists since new mutations are occasionally fixed to give rise to mitochondrial mutants and in some cases these mutants are suppressive. This cannot be simply explained due to a replicative advantage as is seen with yeast suppressivity since [*mi-3*] mitochondria in *Neurospora* do not completely predominate over wildtype in heterokaryons (Lemire and Nargang, 1986) but appear to predominate over [poky] mitochondria in heterokaryons (Gowdrige, 1956). Backer and Birky (1985) found that under selective conditions, intracellular selection for yeast mtDNA molecules containing the erythromycin resistance genotype predominates. It is possible that these observations may be extended to other yeast mitochondrial genes (Backer and Birky, 1985) and perhaps to *Neurospora* mitochondrial genes as well.

Recombination

Evidence for mitochondrial recombination comes from electron microscopic studies on yeast mitochondria (Sena *et al.*, 1986) and observation of extrachromosomal markers in crosses with *S. cerevisiae* and *Chlamydomonas* (Michaelis *et al.*, 1973; Howe 1986). Rothenburg *et al.* (1985) provided direct evidence that intergenomic recombination at the molecular level occurred in homologous regions of two petunia parental genomes in the progeny of a somatic hybrid. Additional studies on yeast zygotes revealed that continual fusion of mitochondria occurs allowing mtDNA molecules from each yeast parent to enter a common pool and undergo multiple rounds of random pairings and genetic exchanges (Foury and Lahaye, 1987).

Sena *et al.* (1986) believe it likely that enzymes for mtDNA recombination are present throughout the life cycle of the cell and are encoded in the nucleus. The latter is supported by the observation of Michaelis *et al.* (1973) who found that when two yeast petite strains were crossed (one carrying erythromycin resistance and the other chloramphenicol resistance), they observed recombinant petites with different suppressive phenotypes from the parental petite strains. These new suppressive petites had mtDNAs of different bouyant densities from the parental petites. The fact that recombination can occur between petite mitochondrial genomes suggests that the enzymes and proteins required for recombination are

synthesized on cytosolic ribosomes and thus are nuclear encoded (Michaelis *et al.*, 1973). The roles of replicative and degradative enzymes in the repair process of mtDNA are unknown for fungi (Backer and Foury, 1985). Although Wintersburger and Blutsch (1976) could find no detectable 3' to 5' exonucleolytic activity associated with mtDNA synthesis in yeast and noted extensive mtDNA degradation after UV irradiation, Uthayashanker and Zassenhaus (1987) have reported a 5' to 3' exonucleolytic activity on double-stranded DNA associated with the mitochondrial nuclease encoded by the yeast nuclear gene *Nuc1*. The *bump1* and *bump2* mutants (involved in an early step of petite formation) have also been found to be involved in mitochondrial recombination in yeast (Maurawiec *et al.*, 1987; Perlman and Butow, 1987). Therefore, it is probable that a replication repair mechanism associated with recombination exists. Studies on mutator strains of yeast have indicated that mismatch repair does not occur in mitochondria (Clayton *et al.*, 1974; Sutherland, 1974; Prakash *et al.*, 1975), but that recombination repair probably is utilized(Prakash *et al.*, 1975). In the same way that nuclear encoded proteins are involved in the splicing of some mitochondrial RNAs (Garriga and Lambowitz, 1984; Garriga and Lambowitz, 1986; Akins and Lambowitz, 1987; Banroques *et al.*, 1987), the enzymes required for mitochondrial recombination are probably provided by nuclear genes or nuclear mitochondrial protein complexes.

Conversion

In their investigation of suppressivity in heterokaryons between [*poky*] and wildtype, Mannella and Lambowitz (1978; 1979) utilized naturally occurring polymorphic forms of mtDNA. These two polymorphic forms of mtDNA were designated type I and type II (Mannella and Lambowitz, 1979). It was determined that recombination between mtDNA molecules occurs frequently in heteroplasmons and that a mixture of different mtDNA molecules is quickly resolved so that one type of mtDNA predominates (either the mutant or the wildtype). It was also shown that two polymorphic insertion sequences are sites of high frequency unidirectional conversion. An insertion of ca. 1200bp exists in the fifth largest mtDNA fragment (generated by EcoRI digestion and designated EcoR1-5) of the type II polymorphic form of *Neurospora crassa* mtDNA relative to the type I form, while the type I polymorphic form of mtDNA carries a ca. 50 bp insert on the EcoR1-9 fragment relative to the type II (Mannella and Lambowitz, 1978). In heterokaryons forced between two strains carrying type I and type II mtDNA, it was found that within the mtDNA of heteroplasmons, EcoR1-5 fragments were

rapidly and completely converted to the type II polymorphic form. This result was confirmed in studies involving different strains by Lemire and Nargang (1986). Mannella and Lambowitz (1979) suggest that this conversion is due to recombination between mitochondrial genomes. They also stated that although these particular fragments do not appear to be associated with the suppressive mitochondrial mutation, the results may indicate that suppressivity is associated with other loci involved in some type of unidirectional conversion that is undetectable by gross restriction analysis (Mannella and Lambowitz, 1979).

Recent DNA sequence analysis of the typeII EcoR1-5 fragment of *N.crassa* mtDNA has shown that the fragment contains the URF1 gene, which is thought to encode a component of the respiratory chain NADH dehydrogenase complex (Burger and Werner, 1985). Sequence analysis of the type II form of EcoR1-5 also revealed the presence of a large class I intron in the URF1 gene. Many class I and II introns have been reported to contain open reading frames, some of which have been noted to encode proteins involved in mRNA splicing.(Waring and Davies, 1984; Michel and Dujon, 1983; Davies *et al.*, 1982; Michel *et al.*, 1982) The URF1 class I intron contains an Unidentified Reading Frame (URF) that is 305 amino acids in length (Burger and Werner, 1985). The significance of this intron and its reading frame is not known although it appears it may have a pattern of inheritance similar to the intron of the 21S rRNA gene of yeast mitochondria (see below).

The behaviour of the insertion in the EcoR1-9 fragment is quite different from that in EcoR1-5. In heterokaryons forced between two strains carrying type I and type IIa mtDNA (wildtype and [*poky*] respectively), EcoR1-9 fragments were converted to the type I insert containing form, at a frequency of about 70% (Mannella and Lambowitz, 1979). However, in similar experiments, but using different *Neurospora* strains ([*mi-3*] (type I mtDNA) and wildtype(type II mtDNA), Lemire and Nargang (1986) found that the EcoR1-9 fragments were converted to the type II form at a frequency of about 70%, a result opposite to the earlier studies.

There are many examples of gene conversion events occurring between mitochondrial genes in yeast and other fungi. Some of these may suggest mechanisms for the conversion events noted in *Neurospora*. For example, the ORF containing, optional intron of the yeast mitochondrial 21S rRNA gene is transferred to non-intron containing strains by a unidirectional conversion process. Strains that carry the intronic gene are called omega$^+$ (ω^+) and those lacking the gene are called

omega $^-$ (ω^-) (Jacquier and Dujon, 1985; Macreadie *et al.*, 1985). Crosses made between the two strains result in nonreciprocal exchange between the two omega alleles. The omega intron is always inserted into the omega$^-$ 21S rRNA gene except in the strains carrying the omega^d and omega^n mutations. The omega^d mutation occurs only in the omega$^+$ strain and has been mapped to a location in the intronic ORF of the (ω^+) 21S rRNA gene. The omega^n mutation only occurs in the omega$^-$ strains and is caused by a single base substitution in the omega $^-$ insertion region. Both mutants prevent the conversion of the omega$^-$ 21S rRNA gene to the omega$^+$ form (Dujon *et al.*, 1985; Jacquier and Dujon, 1985). The omega gene has been found to encode a double strand endonuclease that is specific for the omega$^-$ site. Thus, the intronic ORF of the yeast 21S rRNA gene encodes a protein responsible for its own unidirectional insertion into strains that contain the omega$^-$ insertion site (Jaquier and Dujon, 1985; Macreadie *et al.*, 1985; Zinn and Butow, 1985; Colleaux *et al.*, 1986; Kolodkin *et al.*, 1986; Zinn and Butow, 1986). The similarity of the unidirectional conversion of the EcoR1-5 fragment of *Neurospora* to that of the omega gene in the yeast 21S rRNA intron and the presence of an intron in the URF1 gene of the *N. crassa* EcoR1-5 type II mtDNA fragment suggests that the intron of URF1 may be involved in the unidirectional conversion of the EcoR1-5 fragment.

The *var1* gene in yeast encodes a protein of the small mitochondrial ribosome subunit. The size of the protein is strain dependent. This polymorphism has been found to be due to insertions in the coding region of the *var1* gene (Hudspeth *et al.*, 1982; Butow *et al.*, 1985). All *var1* alleles have a 46 bp GC cluster in the coding region of the gene termed the "common GC cluster". Some of the strains size polymorphisms are due to a second GC insert 146 bp downstream of the "common GC cluster" that has been designated the "a" insert and these strains are designated a$^+$. In a$^+$ X a$^-$ crosses the a$^+$ allele is preferentially recovered whereas it is never recovered in a$^-$ X a$^-$ cross(Hudspeth *et al.*, 1984; Butow *et al.*, 1985). Two other inserts in the coding region also cause protein length polymorphisms. The "b" and "b_p" elements are AT rich inserts. The "b_p" element is a partial version of the "b" element. The former element extends the asparagine cluster in the var1 protein by two additional asparagine residues, the latter extends the cluster by six additional aparagine residues. Unidirectional gene conversion also occurs with the "b" elements but at about 1/15th the frequency of the "a" element. These three polymorphic insertions may be present together or

separately in any given strain (Hudspeth *et al.*, 1984; Butow *et al.*, 1985). Three additional GC clusters (unrelated to the common GC cluster) are found outside the coding region of the var 1 gene. Although they do not affect the var 1 protein, these clusters do appear to be involved in unidirectional conversion (Hudspeth *et al.*, 1984; Butow *et al.*, 1985).

The basidiomycete *Coprinus cinereus*, has two different mitochondrial genomes designated J and H. These mtDNA types also show unidirectional conversion similar to that seen with the *Neurospora crassa* type I and type II mtDNAs. J and H differ with respect to the location of two different 1.23 kb insertions (Economou *et al.*, 1987). J has its insertion located in the cytochrome oxidase subunit 1 gene whereas the insert in the H genome is located approximately 2 kb to one side of the J site. In crosses between the two strains, fourteen out of fifteen independently derived mitochondrial recombinants contained both inserts. The lack of cross hybridization between the two inserts indicate they do not share extensive sequence homology. It was suggested that the "H" and "J" insertions promote conversion events that lead to their own insertions into "H" and "J" insertion sites (Economou *et al.*, 1987).

Aspergillus nidulans and *Aspergillus nidulans* variant *echinulatus* show a similar phenomenon. These two strains have mitochondrial genomes that are identical except for six inserted sequences found in *A. nidulans var. echinulatus*, which account for 16% of its mitochondrial genome (Earl *et al.* 1981). Since these inserts map within mitochondrial genes they are considered optional introns. Strains used from the two species were an *A. nidulans* p-aminobenzoic acid requiring strain carrying an oligomycin resistance mitochondrial gene and a protrophic *A. nid.* var. *echninulatus* strain carryingwt mtDNA. Protoplast fusion between the two species and selection for prototrophs carrying the oligomycin resistant extranuclear drug resistance marker of *A. nidulans* allowed the isolation of recombinant mitochondrial genomes in an *A. Nid* var. *echinulatus* background. Analysis of the recombinant mitochondrial genomes determined that three of the *A. Nid* var. *echinulatus* mitochondrial introns could be transferred to *Aspergillus nidulans* mitochondria (Earl *et al.*, 1981). This directional transfer could be either a result of nuclear background selection, since the nuclear background was that of *A. nidulans* var. *echinulatus*, or it could be due to gene conversion. Since the presence of the the inserts are known to be compatable with an *A. nidulans* nuclear background and similar phenomena occur in *Neurospora* where the nuclear background is the same, Earl *et al.* (1981) favour the latter explanation.

Mitochondrial gene conversion in *Neurospora* has also been noted by Infanger and Bertrand (1986). The [SG-1] cytoplasmic trait of *N. crassa* was transferred to a *N. sitophila* nuclear background through backcrossing of the mutant as the female parent for ten generations to a wildtype *N. sitophila*. Thus the [SG-1] mutant essentially had the mtDNA of *N. crassa* and the nuclear genome of *N. sitophila*. *N. crassa* mtDNA differs from the mtDNA of *N. sitophila* by several DNA restriction fragment length polymorphisms. These insertion polymorphisms are thought to be due to optional introns. When heteroplasmons were formed between *N. sitophila* wildtype and [SG-1] strains, unidirectional conversion was found to occur in the mtDNAs of these heteroplasmons to yield the maximum number of insertions in the cytochrome oxidase subunit 1 and the apocytochrome b genes (Infanger and Bertrand, 1986). This phenomenon may be the result of a mechanism similar to the unidirectional conversion observed with the EcoR1-5 fragments of *Neurospora crassa's* type I and type II mtDNA.

Unidirectional gene conversion has been detected in cytoplasmic organelles of many other organisms. Dron *et al.* (1985) sequenced a fragment of DNA from the chloroplast and mitochondrial genomes of *Brassica oleracea* that both contain a tRNA leu2 gene. The copy in mtDNA is inactive and is described as having been inactivated from the insertion of two short sequences between two sets of direct repeats. These insertion events are believed to have occurred by a gene conversion event. Thus Dron *et al.* (1985) hypothesize that the mitchondrial copy of the tRNA arose from a unidirectional transfer from the chloroplast genome and has been inactivated by the insertion (via a unidirectional gene conversion event) of two fragments into the gene.

Gene conversion has been observed in nuclear genes of several organisms. For example, Swanson and his coworkers (1986) determined that *Gonococci* pilus phase variation, like pilus antigenic variation, can occur by gene conversion of the pilin structural gene in *Neisseria gonorrheae*. Mating type switching in yeast also occurs by a type of gene conversion process (Nasmyth, 1982). A study of immunoglobulin V-region genes in hybridoma cell lines suggests that the diversity of the V region genes (V_H, V_K and V_{lambda}) are generated by gene conversion. (Krawinkle *et al.*, 1986) In chickens it appears that diversification of light chain sequences occurs by a segmental gene conversion mechanism (Reynaud et al., 1987). Chickens construct a newly modified V region in each developing B cell by piecing together (by gene conversion) bits of pseudo-V genes with the rearranged V region of the single functional gene (Maizels, 1987; Reynaud *et al.*, 1987).

Gene conversion was first noted through studies of nuclear recombination in lower eukaryotes such as*Neurospora*, *Ascobolus* and yeast where all products from an individual meiotic event are retained together in an ascus. Through analysis of meiotic recombination it was determined that non-reciprocal recombination may occur relatively frequently (Esser and Kuenen, 1967). This non-reciprocal recombination was referred to as gene conversion. Gene conversion is a recombination mechanism in which genetic information is transferred from a donor to a recipient molecule. Several models have been proposed to explain the mechanism of gene conversion. Meselson and Radding (1975) suggested that the formation of heteroduplex DNA is a normal intermediate in recombination and the process of correcting this heteroduplex leads to conversion. In 1983 Szostak and his colleagues proposed a double-strand break repair mechanism in which the homologue is used as the donor of information (Szostak *et al.*, 1983). This would lead to conversion of deleted information on the gapped chromosome. Hastings (1984) then suggested that double strand break repair is used as a means of resolving the heteroduplex rather than being the initial event in the recombination process. The most recent model by Hamza *et al.* (1986) supports Hasting's view and proposes that there are two types of gene conversion events. Genotype-directed gene conversion assumes that the genotype of one of the molecules creates a preference for itself as donor and donor directed gene conversion occurs when the recipient (of the nick or double strand cut) is preferentially excised and correction occurs using the alternate molecule (the donor) (Hamza *et al.*, 1986). Hamza *et al.* (1986) also indicated when a large heterology is involved in the heteroduplex there should be parity in conversion as long as there is no outside influence on conversion (ie. from a marker close by).

Research Project

In the present study, the sites of unidirectional gene conversion first noted by Mannella and Lambowitz (1978) in the EcoR1-9 and EcoR1-5 fragments were examined by DNA sequence analysis. The EcoR1-5 fragment of type I mtDNA was sequenced to determine whether the URF1 intron was exactly equivilent to the 1200bp insertion of the type II EcoR1-5 and whether it was excised precisely from the type I EcoR1-5 fragment. The sequence of the EcoR1-9 fragments was obtained in order to determine the nature and location of the ca. 50 base pair insert in the type I EcoR1-9 fragment. In order to correlate the conversion events with the transmission of the mutant phenotype, heterokaryons were formed between [*mi-3*] (type I mtiochondrial DNA) and a wildtype (type II mtDNA) strain of *Neurospora crassa* and their DNA was analyzed for frequency and direction of conversion. Cytochrome spectra analysis was performed on the resulting heterokaryons after varying generations of subculturing. Models are proposed to account for the proposed unidirectional conversion at these sites.

MATERIALS AND METHODS

Strains

The *N. crassa* strains used in this study are listed in Table 1.

Table 1

Neurospora crassa strain	Former name	Genotype	Source
NCN10	nic 240	A, nic-1, al-2	Bertrand
NCN20*	Abbot 12 a	A	Lambowitz
NCN45		A,[mi-3],pan2	Lambowitz/Akins
NCN109	HC-2 a	cyb-1,nic-1,al-2,pan-2	Bertrand

*as described by Lemire and Nargang, 1986

Neurospora Stocks

Permanent stocks of *Neurospora* strains were prepared by the procedure of Davis and de Serres (1970). Screw cap tubes (13 x 100 mm) were half filled with silica gel granules (Matheson Coleman and Bell) then heat activated and sterilized in an oven at 180°C for one hour and 30 minutes. The tubes were then cooled with caps tightened. Before use the silica gel tubes were precooled on ice. Reconstituted dry skim milk (10%) was sterilized and 1 ml of milk was added to conidia from 7 to 10 day old conidia slants. The conidia were worked into a suspension and 0.5 ml was added to a cooled tube of silica gel from the bottom of the tube up. The tubes were left on ice for ten minutes and then stored at -20°C.

For routine lab stocks, strains were grown on solid Vogels slants (Davis and de Serres, 1970) at 30°C for 2-4 days and allowed to conidiate in the light at room temperature. The slants were stored at 4°C.

Bacterial Culture Conditions

Escherischia coli strains (table 2) were grown and maintained in L-Broth (Lennox 1955). Strains harbouring recombinant plasmids were grown in media supplemented with the appropriate antibiotics (see appendix). JM103 (Messing 1983) was maintained on Davis minimal medium plus thymine and glucose (see appendix). The strains were grown at 37°C on L-agar plates (see appendix) supplemented with the required antibiotics. When screening for pUC plasmids with inserted DNA fragments, X-gal was added at a concentration of 50 ug/ml and IPTG was added at a concentration of 25 ug/ml. Glycerol stocks were prepared from saturated overnight cultures as described by Maniatis et al.(1982) and stored at -20°C.

Table 2

Strain	Resistance	Genotype	Source	Reference
HB101	Str^R	hsdR-, hsdM-, recA13, ara-14, SupE44, Leu B6, lac Y1, pro A2, thi-1, rpsL20, galK2, xyl-5, mtl-1	Bertrand	Maniatis, 1982
JM83	Str^R	araD(lac-pro), rp5L, thiø80dLacZΔM15,hsdR-	Kelln	Yanish et al., 1985
JM103	Str^R	Δ(pro,lac), supE, thi, strA, end A, sbc B15, F traD36, pro AB, $lacI^q$ zm13	BRL	Messing, 1983
Kurr1226	Chl^R	F-, ara-14, Leu B6, Ton A13, lacY1, tsx-78, supE44, galK2, λ⁻, dcm- 6, his G4, rpsL 136, dam- 13::Tn 9, xyl-5, mtl-1 thi-1, hsdr 2	Kelln	B. Bachman Stock center

Estimation of Conidial Viability

Conidial viability was determined to ensure an equal input of viable conidia into heterokaryon formation. Conidia were suspended in dH_2O and filtered through sterile cheese cloth to remove pieces of mycelium. Conidia concentrations were determined using a haemocytometer. Appropriate dilutions were plated on appropriately supplemented sorbose plates (see appendix). The plates were incubated at 30°C for 1-2 days and viability estimated as the percent conidia growing from the number plated.

Heterokaryons

Heterokaryons were forced by superimposing equal amounts of viable conidia from two different auxotrophic strains of the same mating types onto minimal solid Vogel's conidia medium. The flasks were incubated at 30°C for 2-4 days. The flasks were placed in the light to allow conidiation. A small amount of conidia was passed on to fresh minimal solid Vogel's flasks and slants for successive sub cultures.

Mitochondria and Mitochondrial DNA Isolation

Solid Vogel's medium in erlenmyer flasks was innoculated and incubated 2-3 days at 30°C then allowed to conidiate in the light. Liquid Vogel's medium was innoculated with conidia and incubated at 30°C in a shaker until the culture reached mid to late log phase (usually about 16 hours for wild type *Neurospora* strains and 24 hours or longer for slow growing mutants). Mycelia were harvested by filtering on a Buchner funnel with #4 Whatman filter paper. The mycelial pads were kept on ice or stored at -20°C if the mitochondria were to be isolated at a later date. All procedures were performed at 4°C unless otherwise stated. The mycelia were ground using a mortar and pestle, acid-washed sea sand and grinding buffer (see appendix). About 1.5g sand and 1 ml of buffer was used for each gram of mycelium. Once the mycelium was ground it was suspended in grinding buffer and transferred to centrifuge tubes. The sand and cellular debris were pelleted in an SS-34 rotor at 3,000 RPM for 10 minutes. The supernatant was then transferred to a clean tube and the 3K spin was repeated. The supernatant was then centrifuged for

25 minutes at 12,000 RPM. The pelleted mitochondria were used for cytochrome spectra.(Bertrand and Pittenger, 1969)

The *Neurospora* strains used for isolation of type II and type I mitochondrial DNA were NCN 10 and NCN 20 respectively (see table I). Mitochondrial DNA was isolated as described by Collins et al. (1981) with minor modifications. Mitochondria were purified by flotation gradients as described by Lambowitz (1979) except Tris-HCl was used instead of Tricine KOH.

The mitochondrial pellet was resuspended in 60% sucrose buffer (see appendix). An aliquot of 80% sucrose buffer (see appendix) was added if the mitochondrial suspension was not denser than 55% sucrose buffer (see appendix). The mitochondrial suspension was transferred to SW40 centrifuge tubes (Beckman), three mls of 55% sucrose buffer was layered on top of the mitochondrial suspension and the rest of the SW40 tube was filled with 44% sucrose buffer (see appendix). The tubes were centrifuged at 38,000 rpm for one and a half hours. The mitochondrial band was collected from between the 55% and 44% sucrose layers.

Mitochondria from heterokaryons (between NCN10 and NCN45) were isolated by a step gradient as described by Nargang and Bertrand (1978).The step gradient was constructed by layering 6 ml of 0.8M sucrosebuffer (see appendix) over 6 ml of 2M sucrose buffer (see appendix) in a sorval SS-34 tube. The mitochondrial suspension, in 15% sucrose grinding buffer, was then layered on the 0.8M sucrose and spun at 18,000 rpm for one hour in an SS-34 rotor. The mitochondrial band was collected from the interface of the 2M and 0.8M sucrose buffers.

The sucrose was diluted from the mitochondrial suspension to about 15% by adding 50mM Tris-HCl, 5mM EDTA to the mitochondria and centrifuging at 12,000 RPM for 30 minutes in an SS-34 rotor. A sample of this mitochondria was used for cyotchrome spectra (see below). The bulk of the mitochondrial pellet was resuspended in 2 to 5 ml of 50mM Tris-HCl, 5mM EDTA. 10% SDS was added to a final concentration of 1% SDS. The remaining procedures were performed at room temperature. The sample was extracted twice with water-saturated phenol, once with chloroform:isoamyl alcohol (24:1) (see appendix) and dialyzed overnight against 10mM Tris-HCl[1], pH 7.6 at 4°C, 5mM EDTA. 50 ul of RNase A (see

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[1]In all cases, unless otherwise specified, Tris-HCL used is pH 7.6 at 4°C

appendix) was added and the samples incubated at 37°C for 30 minutes. 1 gram of CsCl was added for every ml of solution and 5 ul of ethidium bromide (10mg/ml) was added for every ml of CsCl solution. This was transferred to quick seal tubes and spun in a VTi65 rotor for 6 hours or more at 54,000 rpm. Both bands (nicked and supercoiled) were collected and the ethidium bromide was extracted with salt-saturated isopropanol (see appendix). The samples were dialyzed overnight against 10mM Tris-HCl, 5mM EDTA . The mitochondrial DNA was stored at -20°C.

Cytochrome spectra

Mitochondrial cytochromes were analyzed using the method of Bertrand and Pittenger (1969). A small amount of isolated mitochondria were centrifuged in a micro-centrifuge for five minutes. The supernatant was discarded and the mitochondria were resuspended in 3 mls of 2.5% deoxycholate, 10 mM Tris-HCl, 5mM EDTA. The samples were centrifuged for five minutes in a micro-centrifuge and the supernatant transferred to two cuvettes. Both tubes were scanned from 650 to 500 nm on a Shimadzu UV-265 spectrophotometer for a base line. The lysed mitochondria in the reference cuvette were then fully oxidized with potassium ferricyanide and lysed mitochondria in the sample cuvette were reduced by addition of sodium dithionite. The samples were then rescanned from 650 to 500 nm to obtain oxidized versus reduced difference spectra.

Restriction Digests

Restrictions digests were carried out following the restriction enzyme suppliers specifications.

Agarose Gel Electrophoresis

Restriction enzyme digests were analyzed by agarose gel electrophoresis. (Maniatis *et al.* 1982) Gels were made to 0.8% agarose in 0.1 M Tris-Borate buffer, pH 8.3, 2 mM EDTA(see appendix). The gels contained ethidium bromide at a concentration of 0.5 ug/ml. (Maniatis *et al.* 1982) The DNA sample was made to 5% glycerol by adding one tenth loading dye (see appendix) just before loading. Electrophoresis was carried out at 25 -100 volts on a constant power supply.

Negative Development of Polaroid 55 Speed Film

The negative was placed in 18% Sodium Sulphite (see appendix) and agitated to remove emulsion (ca. one minute). The negative was then placed in a 21°C water bath for five minutes then dipped in Photoflo (see appendix) for 30 seconds to prevent spotting on the film. Before hanging to dry, the negative was rinsed with deionized water.

Cloning in pUC 19

Purified type II mitochondrial DNA was cut with restriction enzyme EcoR1 and the fragments were shotgun cloned into pUC19 (Yanisch-Perron *et al.*, 1985). The ligated recombinant vector was then transformed into competent cells of the *E. coli* strain JM83. Table 3 shows a list of the recombinant plasmids used in this study.

Table 3

Recombinant Plasmids	Plasmid vector	MtDNA fragment	MtDNA type	*N. crassa* strain source	Transformed *E. coli* strain
pHBE-5 [1]	pBR322	EcoR1-5	II	74A	HB101 Kurr1226
pA5 [2]	pBR325	EcoR1-5	I	NCN 20	JM83 Kurr1226
pAHE-4	pUC19	EcoR1-9	II	NCN 10	JM83 Kurr1226
pAHE-28 [2]	pBR325	EcoR1-9	I	NCN 20	JM83 Kurr1226

1 generously donated by H. Bertrand

2 obtained by screening clones prepared by E. Lemire

Transformation

Ten mls of L-Broth were innoculated with the appropriate *E. coli* strain and grown to saturation overnight at 37°C with shaking. 200 ul of the overnight culture was used to innoculate 25 ml of L-Broth which was shaken in a 37°C incubator until the culture reached mid-log phase (A600 ca. 0.5). The cells were pelleted in a sterile centrifuge tube in an SS-34 rotor at 7,000 RPM for 5 minutes at 4°C. The cells were gently resuspended in 10 mls of transformation mix#1 (see appendix) and again pelleted in an SS-34 rotor at 7,000 RPM for 5 minutes at 4°C. The pellet was then gently resuspended in 5 ml of transformation mix #2 (see appendix) and left on ice for 30 minutes to 24 hours. The cells were then pelleted in an SS-34 rotor at 7,000 RPM for 5 minutes and gently resuspended in a small volume of cold transformation mix #2 (approximately 250 ul per ligation mix). If the amount of ligation mixture to be used was greater than one tenth the volume of competent cells used then the ligation mixture was made to 100mM $CaCl_2$ with sterile 1M $CaCl_2$ before addition to the competent cells. After addition of the ligation mixture, the suspension was incubated on ice for 30 to 60 minutes. Appropriate aliquots (ca. 25 ul to 150 ul) were plated on selective media and incubated overnight at 37°C.

Resistant colonies were screened for recombinant plasmids containing EcoR1-5 fragments and EcoR1-9 fragments from both Type I and Type II mitochondrial DNA. This was done by analysis of plasmid miniprep DNA and filter colony hybridization.

Rapid Plasmid mini-prep

The procedure followed was that of Birnboim and Doly (1979). 10 mls of L-Broth supplemented with the appropriate antibiotic was innoculated with a single resistant colony and incubated overnight at 37°C with shaking. 5 ml of the overnight culture was centrifuged in an SS-34 rotor at 7,000 RPM for 10 minutes at 4°C. The pellet was resuspended in 200 ul of glucose buffer (see appendix) and transferred to a 1.5 ml eppendorf tube. 400 ul of freshly prepared alkaline-SDS solution (see appendix) was added, mixed by inversion and left on ice for 5 minutes. 300 ul of cold 3M NaAc, pH 4.8 was added, mixed by inversion and left on ice for 10 minutes. If a clot formed then the sample was micro centrifuged for five minutes to pellet the clot. If no clot formed then 50 ul of water saturated phenol was added, mixed well, and micro centrifuged for 5 minutes. 750 ul of

supernatant was transferred to a clean eppendorf tube and 450 ul of cold isopropanol was added. The solution was mixed and then placed at -20°C for 5 minutes. The sample was then micro centrifuged for 5 minutes and the supernatant decanted. The pellet was resuspended in 200 ul of TN buffer (see appendix). The tube was filled with 95% ethanol and micro centrifuged for 5 minutes. The supernatant was decanted and the pellet dryed in a vacuum dessicator. The pellet was resuspended in 100 ul of dH_2O and 10 ul was used for each restriction digest. 2 ul of RNase A solution (see appendix) was also added to each digest. The samples were then analyzed by agarose gel electrophoresis.

Large Scale DNA Plasmid Isolation

A 10 ml saturated overnight culture of an *E. coli* strain containing a recombinant plasmid was used to innoculate 500 ml of L-Broth supplemented with the appropriate antibiotic. The culture was placed in a 37°C shaker until it reached late log phase. Then chloramphenicol was added to a final concentration of 170 ug/ml. The culture was left shaking at 37°C overnight. The cells were pelleted in a Sorval GSA rotor at 5,000 RPM for 10 minutes at 4°C. The cells were then resuspended in 2 ml of 25% Sucrose Buffer (see appendix). A small amount of solid lysozyme (ca. 10 mg) was mixed into the cell suspension and left on ice for 10 minutes. 2 ml of 0.25M Na_2EDTA pH 8.0 was added and the mixture was left on ice for 10 minutes. Following this, 4 ml of 4% Triton X-100 was added and the suspension was left on ice for an additional 10 minutes or until it became viscous. The samples were then centrifuged in an SS-34 rotor for 30 minutes at 18,000 RPM and 4°C. The supernatant was decanted into a clean tube and 0.6 volumes of cold isopropanol was added. The samples were mixed and left on ice for 10 minutes. The nucleic acids were pelleted by centrifuging in an SS-34 rotor for 15 minutes at 12,000 RPM and 4°C. The isopropanol was decanted and any isopropanol remaining on the sides of the tubes was removed with tissue. The pellet was resuspended in 5 ml of dH_2O using a pasteur pipet and the insoluble material was pelleted in an SS-34 rotor centrifuged at 10,000 RPM for 15 minutes at 4°C. The supernatant was collected and 1.0 gram of CsCl and 600ul of ethidium bromide (10 mg/ml) was added for every ml of solution. The samples were left for 15 to 30 minutes in the dark at room temperature then centrifuged in a SS-34 rotor for 15 minutes at 15,000 RPM and 4°C. The samples were then transferred to quick-seal tubes and spun for 6 hours or more at 54,000 RPM in a VTi65 rotor or

at 48,000 RPM for 12 hours in the VTi50 rotor at 20°C. The lower band (super coiled DNA) was collected. The ethidium bromide was extracted using salt-saturated isopropanol and the samples dialyzed against 10 mM Tris-HCl, 1 mM EDTA. The plasmid DNA was stored at -20°C.

Large Scale Plasmid DNA Isolation(Alternate)

The procedure followed was that of Maniatis et al. (1982) with some variations. An overnight 10 ml culture of the *E. coli* strain containing one of the recombinant plasmids was used to innoculate 1 litre of L-Broth containing the appropriate antibiotic. The culture was grown in a 37°C shaker overnight. The cells were pelleted in either a GS-3 or GSA rotor at 5,000 RPM for 10 minutes at 4°C. The cells were resuspended in 100 ml of STE buffer (see appendix), divided between four screw cap SS-34 tubes and pelleted in a SS-34 rotor at 7,000 RPM for 10 minutes at 4°C. Each pellet was then resuspended in 10 ml of 10% sucrose buffer (see appendix). 2 ml of freshly prepared lysozyme solution (10mg/ml in 0.25M Tris-HCL) and 8 ml of 0.25M Na2EDTA, pH8.0 were then added to each of the tubes, mixed by inversion, and left on ice for 10 to 30 minutes. 4 ml of 10% SDS was then mixed in gently followed by 6 ml of 5M NaCl. The tubes were then placed on ice for 30 to 60 minutes with occasional mixing. The tubes were centrifuged at 18,000 RPM in an SS-34 rotor for 30 minutes at 4°C to remove high molecular weight DNA. The supernatant was transferred to a clean SS-34 sorval tube and 0.6 volumes of isopropanol added. The tubes were then mixed and left on ice for 10 minutes. The nucleic acids were pelleted by centrifuging at 12,000 RPM for 15 minutes at 4°C. The isopropanol was carefully decanted and any droplets of isopropanol were removed with tissue. The nucleic acids were resuspended in 7 ml of dH_2O using a pasteur pipet. The insoluble material was pelleted by centrifuging at 10,000 RPM for 5 minutes at 4°C. The supernatant volume was measured and 1 gram of CsCl was added per ml of solution. 600 ul of ethidium bromide (10 mg/ml) was added before centrifuging at 15,000 RPM for 15 minutes at 4°C in an SS-34 rotor. The supernatant was transferred to Vti65 quick seal tubes and centrifuged for 6 hours at 54,000 RPM. The lower band (super coiled DNA) was collected and the ethidium bromide extracted using salt-saturated isopropanol. The sample was then dialyzed against 10 mM Tris-HCl, 1 mM EDTA. The DNA was stored at -6°C.

DNA Fragment Isolation and Purification

Recombinant plasmids were cut with the appropriate enzymes following the suppliers recommendations. The fragments were separatedby agarose gel electrophoresis. The mitochondrial DNA fragments were electrophoresed onto NA-45 paper (Schleicher and Schuell Inc. 1984) or DEAE paper (Whatman ion exchange paper: DE81) and extracted according to the suppliers directions.

The NA-45 strip was placed in an 1.5 ml eppendorf tube, washed with dH_2O and then low salt NET buffer (see appendix). 250 ul high salt NET buffer (see appendix) was added to the eppendorf and the sample micro-centrifuged for 5 seconds. The tube was then incubated at 55°C-60°C for 10-45 minutes. The buffer was removed to a clean eppendorf and the NA-45 strip was rinsed with another 50ul of high salt NET buffer. The sample was then extracted twice with water saturated phenol and once with chloroform:isoamyl alcohol (24:1). It was then either dialyzed on a 0.5 μ pore size filter (Sartorius or Milipore) against 10 mM Tris-HCl, 1 mM EDTA or ethanol precipitated and resuspended in an appropriate amount of dH_20 (usually 100 ul).

When DEAE was used the paper was packed loosely in the end of a blue eppendorf tip using a pasteur pipet. The paper was washed with 1 ml of dH_2O once and three times with Low Salt Buffer (see appendix) by forcing the liquid through using a P1000 pipetman. The base of the blue tip was sealed using parafilm and 200 ul of High Salt Buffer (see appendix) was added. After 2 hours at 4°C another 200 ul of High Salt Buffer was added and the buffer collected in an eppendorf tube. The sample was phenol extracted twice with an equal volume of water saturated phenol and once with chlorophorm:isoamyl alcohol (24:1). The DNA sample was then either dialyzed on a 0.5 μ filter (Sartorius or Milipore) against 10 mM Tris-HCl, 1 mM EDTA or ethanol precipitated and resuspended in an appropriate amount of dH_2O (usually 100 ul).

Subcloning into M13

Purified fragments were cut with various restriction enzymes and ligated into the cloning sites of the M13 vectors mp18 and mp19 (Messing 1983). The ligation products were then transfected into competent JM 103 cells (Messing1983).

Dale procedure

Subclones were also produced using the Dale procedure (Dale et al. 1985) to produce deletions in the single-stranded M13 DNA clones.

M13 Transfection

A JM103 colony was used to innoculate a 10 ml tube of DM medium containing thiamine plus glucose and grown to saturation overnight. Two 25 ml LB flasks were innoculated with 200 ul from the fresh JM103 overnight. One flask was grown to mid log phase (A600 ca. 0.5) at 37°C, the other flask was left shaking to saturation for lawn cells. Competent cells and transfection were as described previously (transformation). 25 -150 ul aliquots of the transformation mixture were added to soft agar tubes (see appendix) containing: 0.25 ml lawn cells, 10 ul IPTG (25 mg IPTG/ml of dH_2O) solution and 50 ul X-Gal solution (25 mg X-gal/ml of Dimethyl formamide). The soft agar was then overlayed onto L-Agar plates and incubated overnight at 37°C.

M13 Single-Stranded DNA Isolation

A fresh plaque was picked using a sterile pasteur pipet and the plaque was blown into a 25 ml L-broth flask containing 100 ul of innoculum from a fresh saturated JM103 overnight culture. The culture was grown for 8 to 12 hours at 37°C with rapid shaking. The cells were pelleted by centrifuging in an SS-34 rotor at 15,000 RPM for 30 minutes at 4°C. The supernatant was decanted into a clean tube immediately after centrifugation and 7 ml of 10% PEG solution (see appendix) was added. A phage stock was prepared by removing 1 ml of the supernatant before addition of PEG and storing it at -20°C. The samples were left on ice for 30 minutes or overnight at 4°C. The samples were allowed to become turbid before they were centrifuged in an SS-34 rotor at 12,000 RPM for 20 minutes. The tubes were left to drain at 4°C. Excess PEG was wiped away with a tissue and the phage pellet resuspended in 650 ul of phenol extraction buffer (see appendix). The samples were transferred to eppendorf tubes. 10 ul of 10% SDS and 10 ul of Protease K solution (see appendix) were added to the samples which were then incubated at 37°C for 30 minutes. The sample was then extracted twice with water saturatated phenol and once with chloroform:isoamyl alcohol (24:1) before ethanol

precipitating. The DNA was dryed in a dessicator and resuspended in 50 ul of dH_2O. 2 to 5 ul was analyzed by agarose gel electrophoresis as described above. Mp18 single-stranded DNA was used as a standard to determine size and concentration.

DNA Sequencing

The M13 sub clones were sequenced using the Sanger (Sanger et al. 1977) sequencing method. Single-stranded DNA clones were constructed using M13mp18 or M13mp19 phage vectors. (Yanisch-Perron et al. 1985) The phage DNA was isolated as described above. Single-stranded DNA template (ca. 0.5 to 1.0 ug) was annealed to an appropriate universal M13 primer (Regional DNA Synthesis Laboratory) in 7 mM Tris-HCl, pH 8.0, 0.7 mM $CaCl_2$, 50 mM NaCl (total volume of hybridization was 12 ul). The hybridizations were made either in a pulled capillary tube or a 1.5 ml eppendorf. Annealing was achieved by placing the sample in boiling water and allowing it to cool slowly to at least 37°C. The sample was then transferred to a well in a Nunc microwell plate (60 wells per plate with lid). 1 ul of dithiothreitol and 1 ul of the radioisotope were added. The raidoisotopes used were either alpha ^{32}P-dATP (> 600 Ci/mmol) or alpha ^{35}S-dATP (> 1000 Ci/mmol).

The reaction was started by addition of 1 ul of Klenow fragment (1 unit/ul). The reaction was mixed well and 3 ul was transferred to four adjacent wells. 3 ul of A, C, G, or T mix (see appendix) was added to the appropriate well, mixed, and left in an air incubator for 10 minutes at 45-55°C. 1.5 ul of chase mix (see appendix) was then added and the samples were left incubating for another 10 minutes at 45-55°C. (It should be noted that for ^{35}S reactions the samples could be left for 20 minute incubation periods in order to obtain better incorporation of the radioisotope.) Formamide stop dye (see appendix) was added and the samples transferred to eppendorfs. The samples were denatured by placing them in boiling water for 5 minutes. The samples were then loaded onto a gel of 6% acrylamide (prepared form 40% acrylamide/bis-acrylamide (19:1) (w/w) stock), 8.33 M Urea, 0.1 M Tris-Borate, pH 8.0, 2 mM EDTA. The samples were run at a constant power setting of 20-45 watts for a 40 cm X 20 cm X 0.25 mm gel. For longer gels the wattage was adjusted appropriately.

Autoradiography

^{35}S gels were dryed in a vacuum dryer (Bio-Rad model 483 slab dryer) at 80°C. ^{32}P and ^{35}S gels as well as Southern transfer and Filter colony hybridization blots were exposed to Kodak XAR-5 film in film holders or cassetes. ^{32}P gels and hybridization blots were kept at -20°C. After sufficient exposure time (16 to 48 hours) the film was developed manually using Kodak developer and fixer following the suppliers instructions.

DNA Sequence Analysis

DNA sequences were analyzed with the Beckman Microgenie program. The tRNA search was performed using the Staden (1980) tRNA search program.

Southern transfer and Filter colony hybridization

The probe was labeled by the technique of Feinberg and Vogelstein (1983,1984). The DNA (0.2 ug to 0.5 ug) was brought up to a volume of 32 ul in an eppendorf tube. The sample was boiled for 2 minutes then placed on ice and 2ul of BSA (bovine serum albulmin, 10 mg/ ml) was added. 10 ul of OLB (see appendix) and 5 ul of alpha ^{32}P-dCTP was added. The labeling reaction was started by adding 2 units of Klenow. The sample was placed at 37°C for 2 hours to overnight. Stop buffer was added (see appendix) and the sample run on a sephadex G50 column to remove the free nucleotides. The sample was counted and counts per minute (cpm) per ug of DNA was calculated for addition to the hybridization solution.

Recombinant plasmid was cut with the appropriate restriction enzyme following the supplier's recommendations and the fragments were electrophoretically separated on a 0.8% agarose gel as described previously. The DNA was transferred to biodyne membrane and hybridized to radioactive probe following the procedures recommended by the suppliers (ICN Biomedicals Inc. 1986). The agarose gel was placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes on a slow shaker. The gel was then neutralized by placing it in neutralizing solution (3M Na acetate, pH 5.5) on a slow shaker for 30 minutes. The gel was then placed on plastic wrap and a piece of gel sized biodyne membrane was placed on top of the gel. A stack of paper towels and and a 1 kilogram weight

were placed on top of the gel for 12 hours to overnight to allow transfer of the DNA to the biodyne membrane. The membrane was then baked at 80°C for 1 hour.

The membranes for filter colony hybrization were prepared by placing sterile biodyne paper on L-broth plates supplemented with the appropriate anitbiotics. The transformed colonies were patched onto the membrane and incubated overnight (or transferred to chloramphenicol plates for amplification). The biodyne membrane with colonies on its surface was then soaked with denaturing solution (1.5 M NaCl, 0.5 M NaOH) in a petri dish for 5 minutes and then with neutralizing solution (3M Na acetate, pH 5.5) for 5 minutes. The biodyne was then rinsed with dH_2O and baked at 80°C for 1 hour.

The prepared biodyne membranes were then prehybridized by sealing the membrane in a plastic bag with prehybridization solution (see appendix) for a minimum of 1 hour in a 65°C water bath. The prehybridization solution was discarded and hybridization was obtained by sealing more prehybridization solution and the prepared probe with the membrane in the plastic bag. The sample was then left hybridizing a minimum of 16 hours in a 65°C water bath. The membrane was then rinsed with wash buffer (5 mM Naphosphate, pH 7.0, 1 mM EDTA, 0.2% SDS) by shaking on a slow shaker and checking radioisotope counts every 30 minutes until the background was clean. The membrane was then sealed in a plastic bag and set up for autoradiagraphy.

RESULTS

Clones of Mitochondrial DNA for Further Study

The two polymorphic forms of *N. crassa* mitochondrial DNA that are of concern in this thesis are type I and type II. The two forms are routinely distinguished by their electrophoretic pattern of restriction fragments following EcoRI digestion. (Fig. 1) As first described by Mannella and Lambowitz (1978, 1979) type II mitochondrial DNA contains a ca. 1200 base pair insertion in the EcoR1-5 fragment relative to type I. Type I mitochondrial DNA contains a ca. 50 base pair insertion in the EcoR1-9 fragment relative to type II. (see Fig. 2) The *Neurospora crassa* strains utilized to isolate type I and type II mitochondrial DNA were NCN 20 and NCN 10 respectively (see table 1 in Materials and Methods).

In order to obtain large amounts of the EcoR1-5 and EcoR1-9 fragments from the two types of mitochondrial DNA, all four fragments were cloned into bacterial plasmid vectors. Unscreened isolates from a library of type I mitochondrial DNA EcoR1 fragments cloned into plasmid pBR325 were provided by E. Lemire (Department of Genetics, University of Alberta). These were digested with EcoR1 and electrophoresed on an agarose gel . Purified type I mitochondrial DNA digested with EcoR1 was electrophoresed on the same gel to serve as a size standard for the fragments carried in the various clones. (Fig. 3) Plasmid A5 (Fig. 3, lane E) was found to contain the EcoR1-5 fragment of type I mitochondrial DNA and chosen for large scale isolation of this fragment. A plasmid, designated AHE-28 (Fig. 3, lane L) , was found to contain the EcoR1-9 fragment of type I mitochondrial DNA. It was later found that this clone also contained the small EcoR1-11 fragment. AHE-28was chosen for large scale isolation of the EcoR1-9 fragment.

Because of possible nuclear DNA contamination in the isolation of the type II EcoR1-9 fragment, it was deemed necessary to confirm the identity of the EcoR1-9 fragment in AHE28 and subsequently in the plasmid chosen for type II EcoR1-9 large scale isolation (see below). Therefore, type I EcoR1-9 fragment was purified from an agarose gel in which Type I mtDNA digested with EcoR1 had been electrophoresed. The isolated fragment was then labelled with ^{32}P. Southern analysis, utilizing the labelled type I EcoR1-9 fragment as probe, confirmed that AHE-28 contained the type I EcoR1-9 fragment.(Fig. 4 and 5, lanes C and D) The

EcoR1-9 fragment was also cloned into the phage vector M13mp19 in both orientations. (Fig. 4 and 5, lanes F and G)

A clone of the type II version of EcoR1-5 carried in plasmid pBR322 was kindly provided by H. Bertrand (Department of Biology, University of Regina). In order to obtain a clone of the type II EcoR1-9 fragment, type II mitochondrial DNA was isolated from strain NCN10 (table 1) and digested with EcoR1. The fragments were "shotgun" cloned into plasmid pUC19 that had also been digested with EcoR1. The ligation products were transformed into strain JM83 and plated on L-agar containing ampicillin, X-gal and IPTG. White colonies were patched onto biodyne membrane and examined by filter colony hybridization as described in Materials and Methods. The lysed colonies were probed with ^{32}P-labelled EcoR1-9 fragment purified from plasmid AHE-28. Several colonies were chosen for further analysis. (Fig. 6) Their plasmid DNA was isolated, digested with EcoR1, and analyzed by agarose gel electrophoresis. (Fig. 7) The DNA was further examined by southern analysis using ^{32}P-labelled EcoR1-9 fragment purified from plasmid AHE-28 as the probe. (Fig. 8) A plasmid, designated AHE-4, was chosen for large scale isolation and analysis of the type II EcoR1-9 fragment.

Table 4 summarizes the recombinant plasmids used in this study. Figure 9 shows the two types of mitochondrial DNA and each of the recombinant plasmids used in the study.

Table 4

Recombinant Plasmid	DNA Type	EcoR1 Fragment
AHE 28	Type I	EcoR1-9
A5	Type I	EcoR1-5
AHE4	Type II	EcoR1-9
HBE4	Type II	EcoR1-5

Figure 1

Agarose Gel of Type I and Type II *Neurospora crassa* Mitochondrial DNA Digested With EcoR1

NCN 10 and NCN 20 mtDNA was cut with EcoR1 and electrophoresed in a 0.8% agarose gel. Lane 1 contains NCN 20 mtDNA and Lane 2 contains NCN 10 mtDNA.

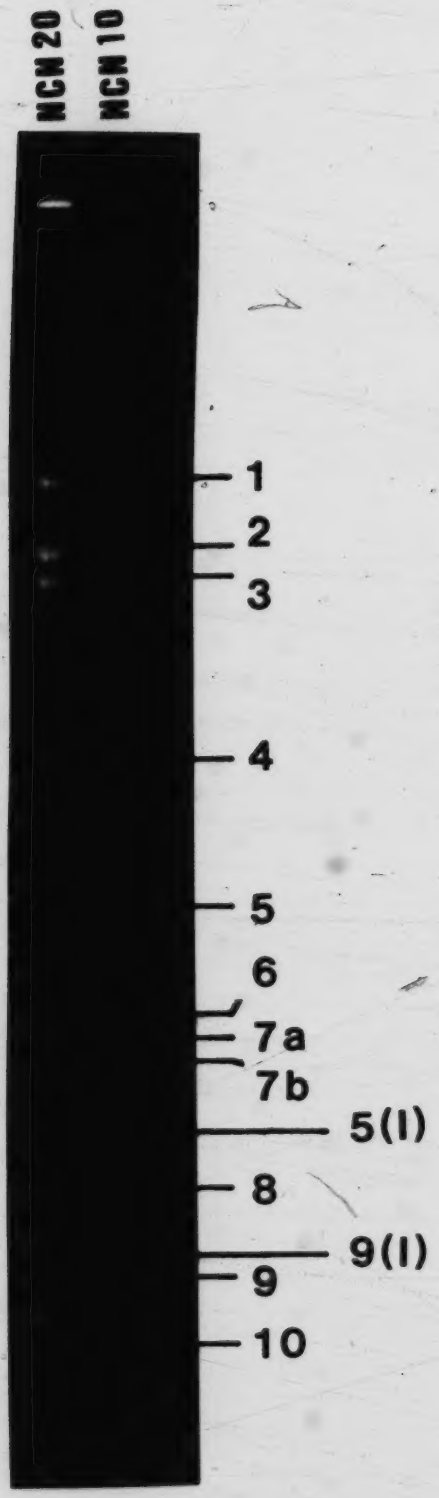

MCM 20
MCM 10
1
2
3
4
5
6
7a
7b
5(I)
8
9(I)
9
10

Figure 2

Map of *Neurospora Crassa* Mitochondrial DNA

Neurospora crassa mt DNA map adapted from Burger and Werner (1985). The insertions found in the EcoR1-9 and EcoR1-5 fragment of the type I and type II mtDNA (respectively) are indicated on the EcoR1 restriction map of the inner circle. Mitochondrial genes are indicated on the outer circle. Exons are indicated by black boxes. Unidentified intronic reading frames are indicated by grey boxes. tRNA genes are indicated by thin bars. COI, COII, and COIII: genes encoding cytochrome oxidase subunits 1, 2 and 3 respectively. COB: gene encoding apocytochrome b. ATPase 6: gene encoding subunit 6 of mitochondrial ATP synthase. ATPase 8: gene encoding subunit 8 of mitochondrial ATP synthase. MAL: ORF with homology to subunit 9 of mitochondrial ATP synthase. s-rRNA: gene encoding the mitochondrial small ribosomal subunit. l-rRNA: gene encoding the mitochondrial large ribosomal subunit. putative S5: small mitochondrial ribosomal subunit protein encoded in l-rRNA intron. URFs: unidentified reading frames- some of which have homology to mammalian URFs known to encode subunits of the NADH dehydrogenase complex.

Neurospora crassa Mitochondrial DNA

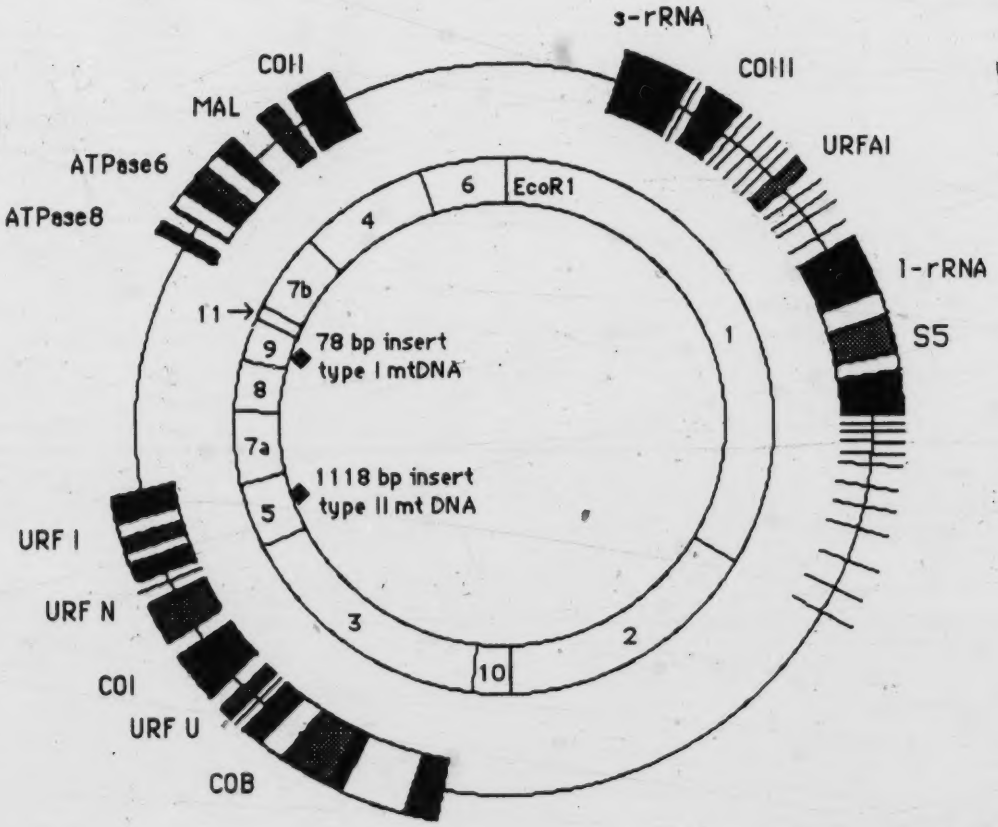


Figure 3

Agarose Gel of Type I Mitochondrial DNA EcoR1 Fragment Library Clones Digested With EcoR1

The DNA was electrophoresed in a 0.8% agarose gel. Lanes A and B: EcoR1 cut type I mtochondrial DNA. Lanes C and D: EcoR1 cut type II mitochondrial DNA. Lanes E to L: Clones from type I mitochondrial DNA EcoR1 library in pBR325 digested with EcoR1. Lane M: EcoR1 cut pBR325.

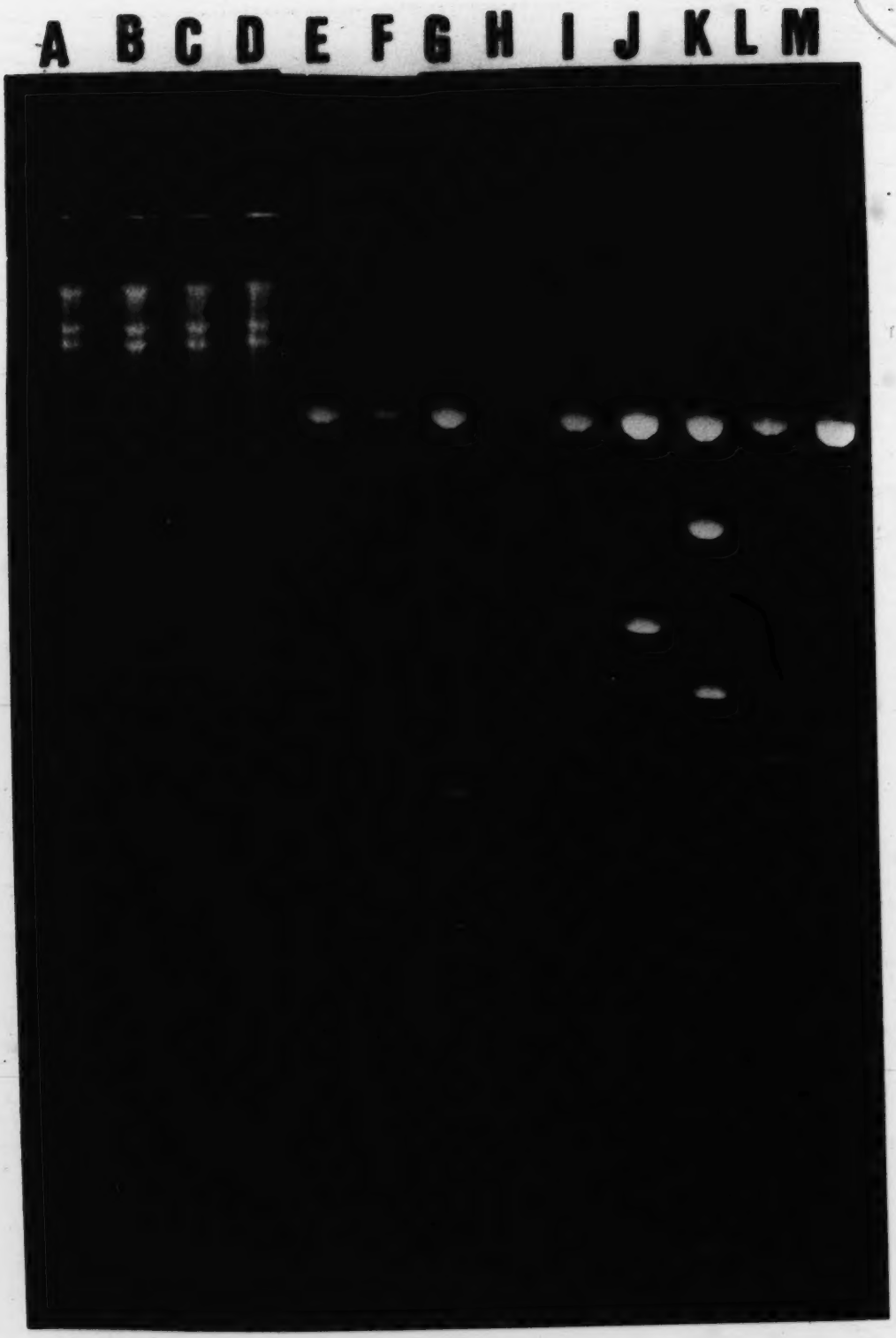

A B C D E F G H I J K L M

Figure 4

Agarose Gel of Plasmid AHE-28, EcoR1-9 Type I Fragment and Mp19 Clones Containing Type I EcoR1-9

The DNA was electrophoresed in a 0.8% agarose gel. Lane A: Lambda DNA cut with EcoR1 and Hind III for marker DNA, Lane B: pBR325 cut with EcoR1, Lane C: Plasmid AHE-28 cut with EcoR1, Lane D: purified type I EcoR1-9 fragment, Lane E: type I mitochondrial DNA cut with EcoR1, Lane F and Lane G: single stranded M13 mp18 vector containing the type I EcoR1-9 fragment inserted in opposite orientations (clones GBG2 and GBG7 respectively).

A B C D E F G

Figure 5

Southern Blot IdentifyingPlasmid AHE-28,
EcoR1-9 Type I Fragment and M19 Clones Containing Type I EcoR1-9

The DNA in the gel shown in Fig. 3 was transferred to biodyne membrane and probed with ^{32}P labeled EcoR1-9 fragment. Type I EcoR1-9 fragment was purified from an agarose gel in which Type I mtDNA digested with EcoR1 had been electrophoresed and was labelled with ^{32}P. The blot was autoradiographed as described in Materials and Methods. Lane A- Lambda DNA cut with EcoR1 and Hind III, Lane B- PBR325 cut with EcoR1, Lane C- Plasmid AHE-28 cut with EcoR1, Lane D- purified type I EcoR1-9 fragment, Lane E- type I mitochondrial DNA cut with EcoR1, Lane F and Lane G- single stranded M13 mp18 vector containing the type I EcoR1-9 fragment inserted in opposite orientations (GBG2 and GBG7 respectively).

A B C D E F G

Figure 6

Filter Colony Hybridization of Shotgun Cloned Type II Mitochondrial DNA Fragments - Selection for Cloned EcoR1-9 Type II Fragments

E. coli colonies containing recombinant Puc19 vector containing inserts from type II mitochondrial DNA were grown on biodyne membrane that had been placed on selective media. The colonies were lysed and the membrane prepared for filter colony hybridization as described in Materials and Methods. ^{32}P labeled type I EcoR1-9 fragment isolated from plasmid AHE-28 was used to probe for the colonies carrying type II EcoR1-9 fragments. Colonies labeled 1 through 9 were those chosen for further analysis as described in the text.

Figure 7

Agarose Gel of the Potential Type II
EcoR1-9/pUC19 Recombinant Plasmids

The EcoR1 cut DNA was electrophoresed in a 0.8% agarose gel. Lanes A, F, and L: Type II mitochondrial DNA cut with restriction enzyme EcoR1, Lanes B,C,D,E,G,H,I,J, and K: represent the plasmid DNA from the chosen isolates 1 through 9 respectively as shown on Fig. 5. This gel was used for transfer to Biodyne membrane for southern analysis.(see Fig 7)

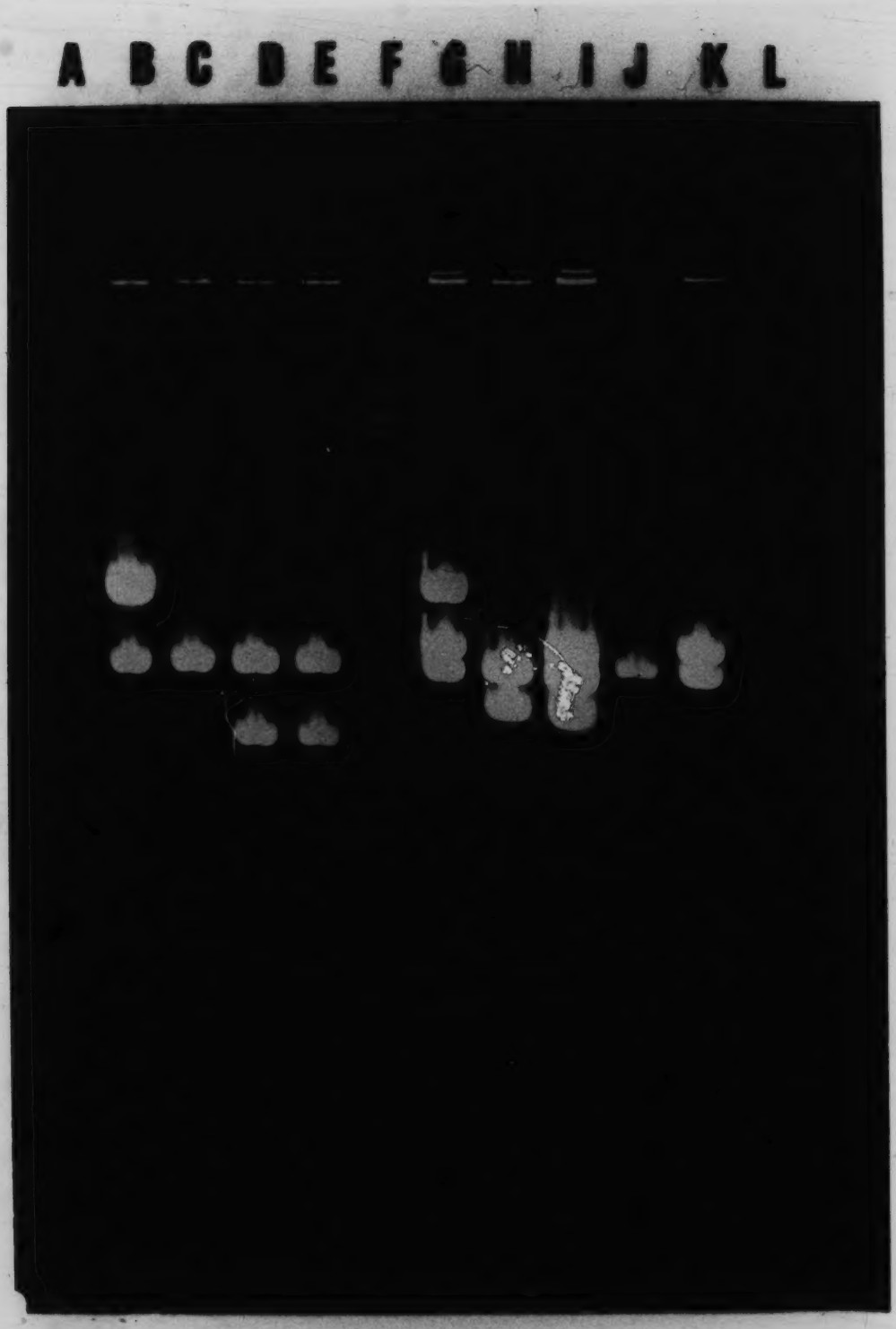

A B C D E F G H I J K L

Figure 8

Southern Blot Identifying Plasmid AHE-4
as Carrying the Type II EcoR1-9 Fragment

Plasmid DNA isolated from the nine positive colonies labeled in figure 4 were cut with the restriciton enzyme EcoR1 and electrophoresed in a 0.8% agarose gel shown in Fig 6. The DNA was transferred to biodyne membrane as described in materials and methods. ^{32}P labeled type I EcoR1-9 fragment isolated from plasmid AHE28 was used as the probe to identify those recombinant plasmids that contain the type II EcoR1-9 fragment. Lanes A, F, and L: Type II mitochondrial DNA cut with restriction enzyme EcoR1, Lanes B,C,D,E,G,H,I,J, and K: represent the plasmid DNA from the chosen isolates 1 through 9 respectively (Fig. 5). Isolate 4 (lane E) was chosen for isolation and purification of type II EcoR1-9 fragments. The plasmid was named AHE-4.

A B C D E F G H I J K L

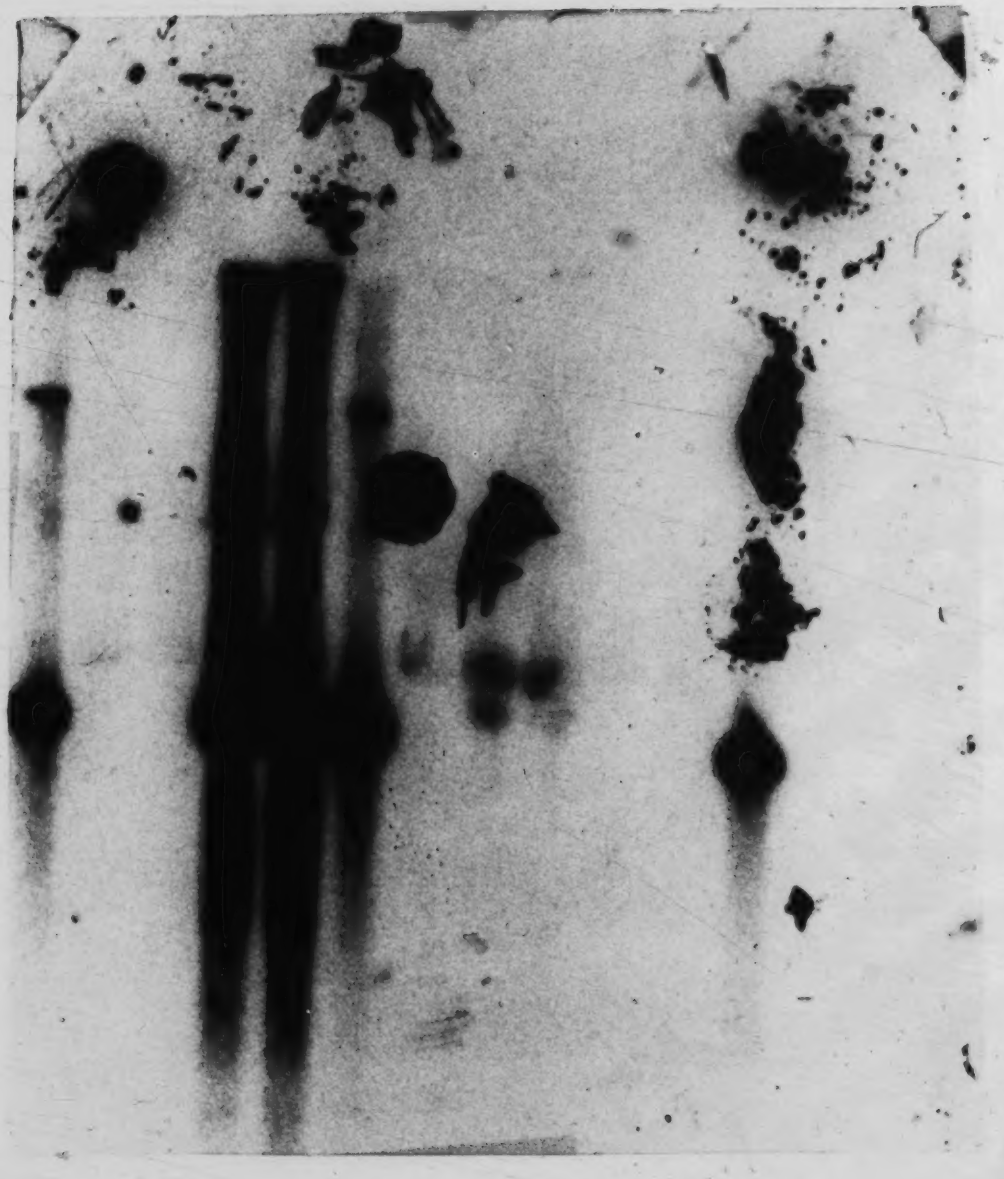
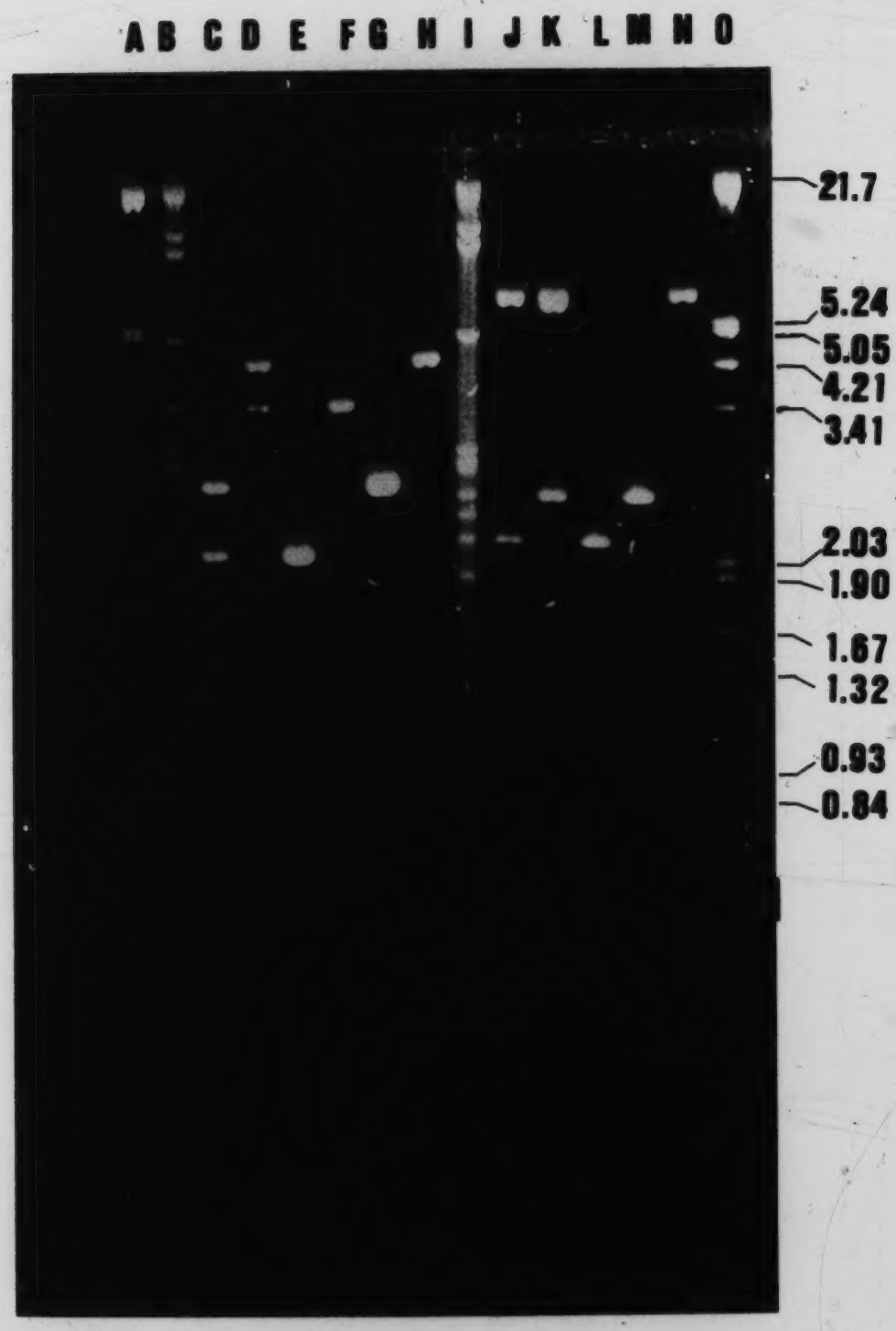


Figure 9

Agarose Gel of Recombinant Plasmids and Isolated DNA Fragments

DNA from recombinant plasmids, and from type I and type II mitochondrial DNA were digested with EcoRl. These were electrophoresed in a 0.8% agarose gel along with purified EcoR1-5 and EcoR1-9 fragments. EcoR1/Hind3 cut Lambda phage DNA was used as a size marker.

Lane A: Lambda phage DNA digested with EcoRl/Hind3, Lane B: type II mt DNA, Lane C: pAHE-4, Lane D: pHBE-5, Lane E: type II EcoRl-9 fragment, Lane F: type II EcoRl-5 fragment, Lane G: Puc l9, Lane H: PBR322, Lane I: type I mt DNA , Lane J: pAHE-28, Lane K: pA5, Lane L: type I EcoRl-9 fragment, Lane M: type I EcoRl-5 fragment, Lane N: pBR325, Lane O: Lambda phage DNA digested with EcoR1/Hind3.


A B C D E F G H I J K L M N O
21.7
5.24
5.05
4.21
3.41
2.03
1.90
1.67
1.32
0.93
0.84

DNA Sequence Analysis of Type I and Type II EcoR1-9 Fragments

In order to determine the nature of the insert in the type I EcoR1-9 fragment relative to the type II fragment, both type I and type II EcoR1-9 fragments were subcloned into the M13 vectors mp18 and mp19. This facilitated the isolation of single-stranded template for DNA sequence analysis. The EcoR1-9 fragments of type I and type II mitochondrial DNA were isolated and purified from the plasmids AHE-28 and AHE-4 respectively. Various restriction enzymes that had a restriction site of 6 base pairs and/or digested the fragments into five or less fragments were initially utilized for subcloning of the EcoR1-9 fragments into M13 mp18 and mp19 vectors. Other restriction enzyme sites were chosen from the sequence as it was obtained. Figs. 10 and 11 show the linear restriction maps and DNA sequencing strategies for the EcoR1-9 type I and type II fragments respectively. Both strands of the type I EcoR1-9 fragment were sequenced entirely. A complete single-stranded sequence of the type II EcoR1-9 fragment was obtained with double-stranded sequence obtained in the repeat region (see below) and in those regions where the sequence was unreliable due to compression effects. The two fragments were found to have identicle nucleotide sequence except for the presence of an extra copy of a 78 base pair repeat (Fig. 12) found in the type I form of EcoR1-9. (Fig. 13) One sequence gel for both the type I and type II fragments was obtained which could be read completely through the repeat regions. These gels confirmed that the type I EcoR1-9 fragment contains three copies of the 78 base pair repeat and the type II form contains only two copies of the repeat. This extra repeat is the reason for the polymorphic size difference between the two fragments. Fig. 13 gives the complete nucleotide sequence of the type I EcoR1-9 fragment. The three copies of the repeat start at nucleotides 112, 190, and 268 and end at nucleotide 346.
The nucleotide content of *Neurospora crassa* mitochondrial DNA has been reported as 40% G+C. (Bernard *et al.*, 1975; Terpsta *et al.*, 1977) The EcoR1-9 fragments were found to be typical of *Neurospora* mitochondrial DNA as their G+C content was found to be 38% (table 5). However, the insert region was found to be more A+T rich (70.5%) than *Neurospora* mitochondrial DNA in general (60%).

Figure 10

DNA Sequencing Strategy Used for DNA Sequence Determination of the Type I EcoR1-9 Fragment

Figure 9 shows a partial restriction endonuclease map and DNA sequencing strategy for the Type I EcoR1-9 fragment of *Neurospora crassa* mtDNA. The horizontal arrows indicate the direction and extent of sequencing

Figure 10

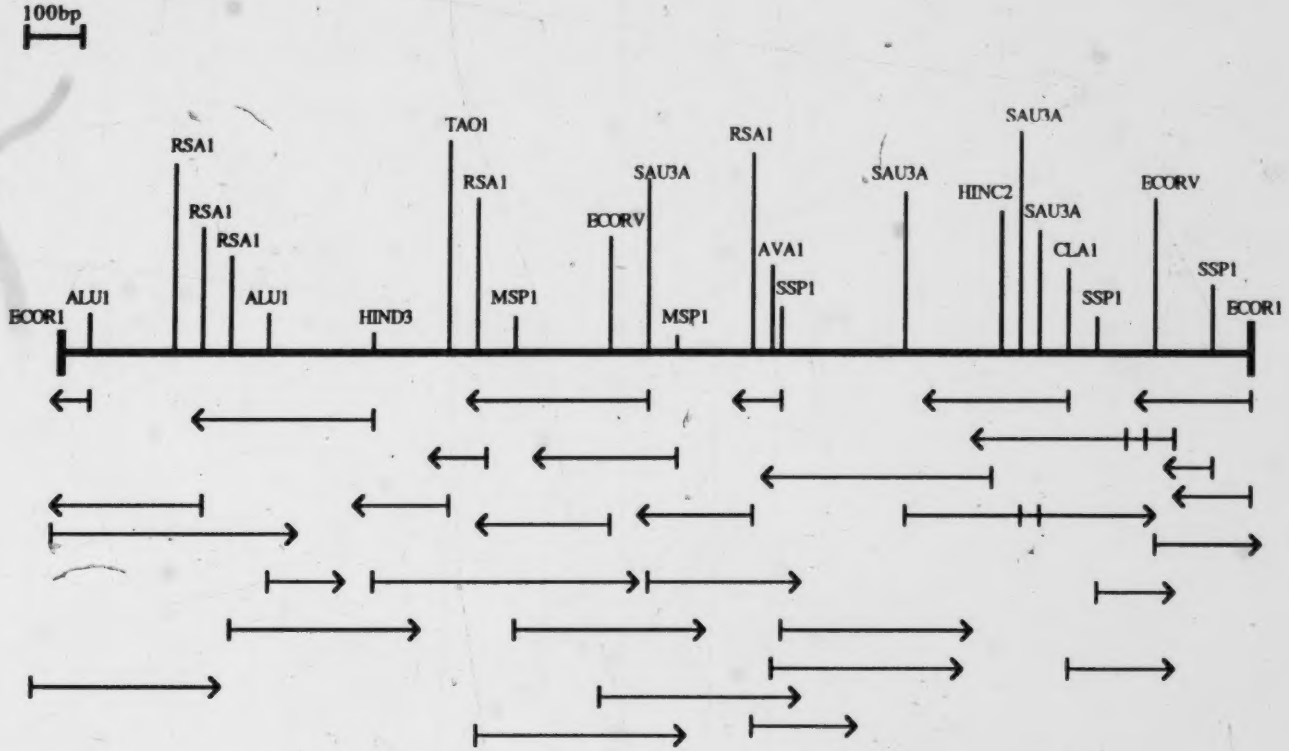

100bp
BCOR1
ALU1
RSA1
RSA1
RSA1
ALU1
HIND3
TAO1
RSA1
MSP1
BCORV
SAU3A
MSP1
RSA1
AVA1
SSP1
SAU3A
HINC2
SAU3A
SAU3A
CLA1
SSP1
BCORV
SSP1
BCOR1

Figure 11

DNA Sequencing Strategy Used for DNA Sequence Determination of the Type II EcoR1-9 Fragment

Figure 10 shows a partial restriction endonuclease map and DNA sequencing strategy for the Type II EcoR1-9 fragment of *Neurospora crassa* mtDNA. The horizontal arrows indicate the direction and extent of sequencing

Fig 11

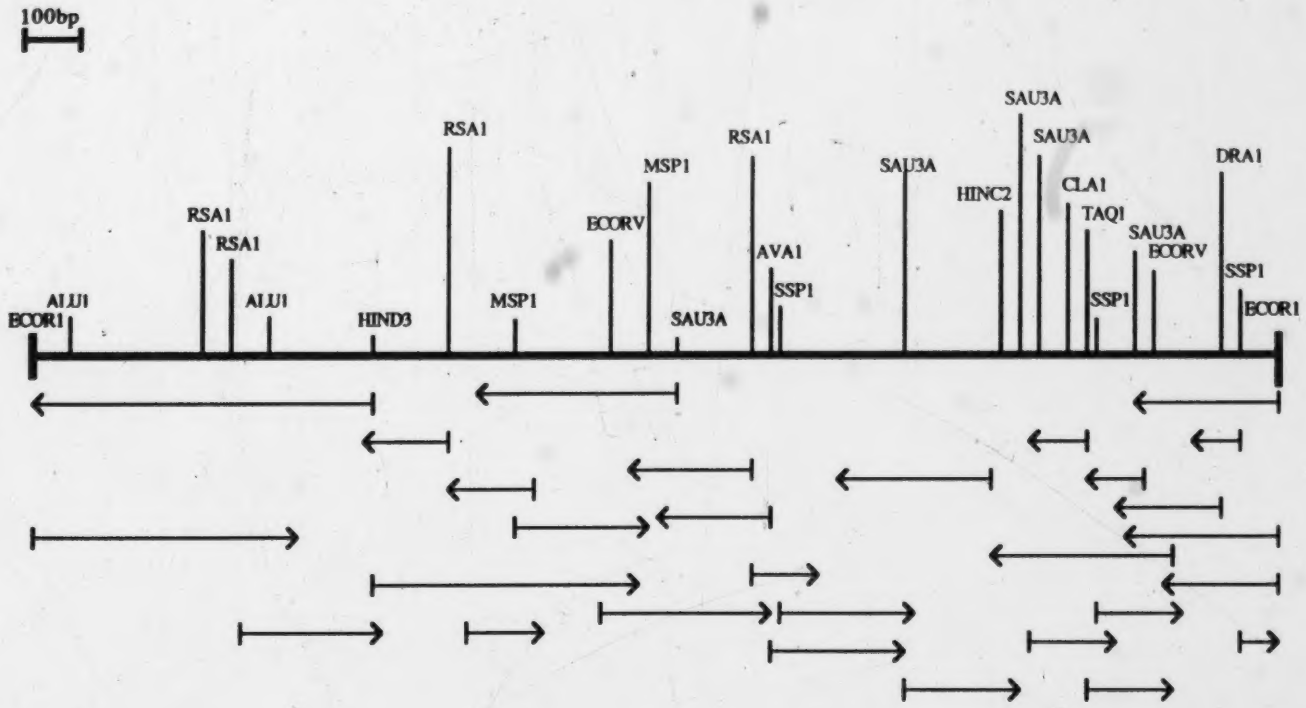

100bp
ECOR1
AIJI
RSA1
RSA1
AIJI
HIND3
RSA1
MSP1
ECORV
MSP1
SAU3A
RSA1
AVA1
SSP1
SAU3A
HINC2
SAU3A
SAU3A
CLA1
TAQ1
SSP1
SAU3A
ECORV
DRA1
SSP1
ECOR1

Figure 12

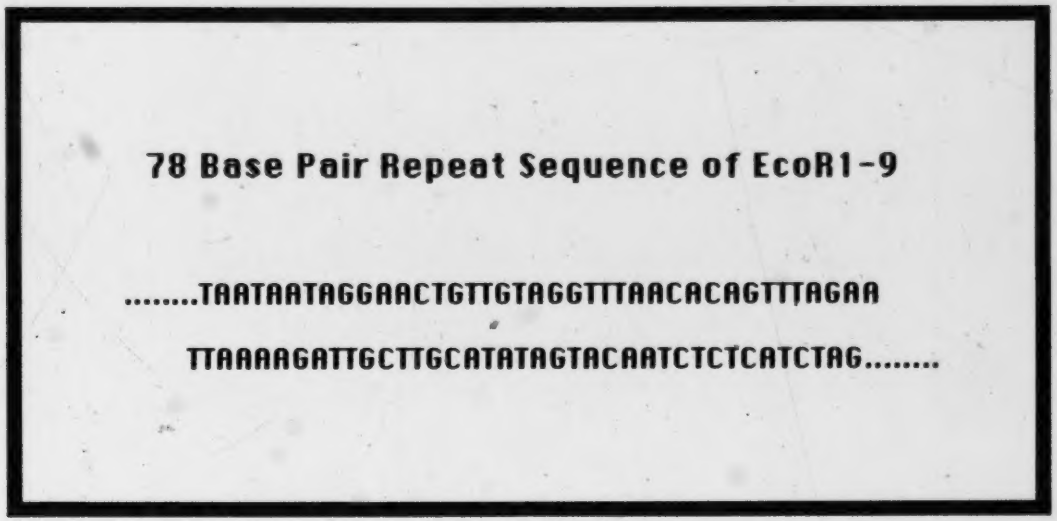

78 Base Pair Repeat Sequence of EcoR1-9
.....TAATAATAGGAACTGTTGTAGGTTTAACACAGTTTAGAA
TTAAAAGATTGCTTGCATATAGTACAATCTCTCATCTAG.....

Figure 13

DNA Sequence of Type I EcoR1-9 Fragment

The complete sequence of type I EcoR1-9 fragment. The 78 base pair repeat region begins at nucleotide 112 and ends at nucleotide 346. The second and third repeats begin at nucleotide 190 and 268 respectively.

```
                                                                    50
GAATTCTAGT GGATTTTTAT CTGATCAAGG ATTAGCTAAT TTCTTATATA
CTTAAGATCA CCTAAAAATA GACTAGTTCC TAATCGATTA AAGAATATAT

                                                                   100
AACTCAAACA ACCACCTCCA AGGAATAAAC AACTACAATT ATACCCCCCT
TTGAGTTTGT TGGTGGAGGT TCCTTATTTG TTGATGTTAA TATGGGGGGA

                                                                   150
CATGCAGCAA ATAATAATAG GAACTGTTGT AGGTTTAACA CAGTTTAGAA
GTACGTCGTT TATTATTATC CTTGACAACA TCCAAATTGT GTCAAATCTT

                                                                   200
TTAAAAGATT GCTTGCATAT AGTACAATCT CTCATCTAGT AATAATAGGA
AATTTTCTAA CGAACGTATA TCATGTTAGA GAGTAGATCA TTATTATCCT

                                                                   250
ACTGTTGTAG GTTTAACACA GTTTAGAATT AAAAGATTGC TTGCATATAG
TGACAACATC CAAATTGTGT CAAATCTTAA TTTTCTAACG AACGTATATC

                                                                   300
TACAATCTCT CATCTAGTAA TAATAGGAAC TGTTGTAGGT TTAACACAGT
ATGTTAGAGA GTAGATCATT ATTATCCTTG ACAACATCCA AATTGTGTCA

                                                                   350
TTAGAATTAA AAGATTGCTT GCATATAGTA CAATCTCTCA TCTAGGTTTT
AATCTTAATT TTCTAACGAA CGTATATCAT GTTAGAGAGT AGATCCAAAA

                                                                   400
ATTTTATTAG CTTTAAGTGT TTCCACACAA AGTGTGGAGT CCACACAAGC
TAAAATAATC GAAATTCACA AAGGTGTGTT TCACACCTCA GGTGTGTTCG

                                                                   450
ATTTATATTT TGGGAGGAGT GAAATTACAG GGAGATTAAA ATAGTTTAAT
TAAATATAAA ACCCTCCTCA CTTTAATGTC CCTCTAATTT TATCAAATTA

                                                                   500
CTGTAAGAAC ATTAAGATGG CGATTCCACT CGGCCCCCCC TCCAGCGAAG
GACATTCTTG TAATTCTACC GCTAAGGTGA GCCGGGGGGG AGGTCGCTTC

                                                                   550
CTGGAGGGGG TAGCGAGTGA AAGCTTTTAA TAAAGTTGGA TTGCCATGAC
GACCTCCCCC ATCGCTCACT TTCGAAAATT ATTTCAACCT AACGGTACTG

                                                                   600
CGCTTAGAGC GTTGGTCCCT ATCTTCCTCA GTAGTTTACT GCTGAGGAAG
GCGAATCTCG CAACCAGGGA TAGAAGGAGT CATCAAATGA CGACTCCTTC

                                                                   650
GTAGAGCAGT CAGCAAAGAG GACGGACCGT AAAGCCATAC ATCGAGGGAA
CATCTCGTCA GTCGTTTCTC CTGCCTGGCA TTTCGGTATG TAGCTCCCTT

                                                                   700
TAGTAAACCC CAGCTACCCT ACAGATTTTA ATCTGTAGGG TAGCCTTATG
ATCATTTGGG GTCGATGGGA TGTCTAAAAT TAGACATCCC ATCGGAATAC

                                                                   750
GAATAGAGGA AAAAATTTTC CTAACGTAAA TACCTTAATG CCTCCCAGGA
CTTATCTCCT TTTTTAAAAG GATTGCATTT ATGGAATTAC GGAGGGTCCT
```

```
                                                                    800
GATAAGTATC ATTGATGTTA TCCTAGTACC TATTTTCACC TATAATCACT
CTATTCATAG TAACTACAAT AGGATCATGG ATAAAAGTGG ATATTAGTGA

                                                                    850
TGTCGCTGAT AAGCTAGCGC AGGTGCGCTG GTGCCTTGAC CCCGGATGGT
ACAGCGACTA TTCGATCGCG TCCACGCGAC CACGGAACTG GGGCCTACCA

                                                                    900
CGTATTTTTC CTACTATTGA CGAGGGTATT CAATATCTAG GAGAAATTTC
GCATAAAAAG GATGATAACT GCTCCCATAA GTTATAGATC CTCTTTAAAG

                                                                    950
GATTTCTCTA GGTAATATGA ACCAGGCTTT GGCCCACTCC ATTGGGTATT
CTAAAGAGAT CCATTATACT TGGTCCGAAA CCGGGTGAGG TAACCCATAA

                                                                   1000
ATCGGTTGAA AAGAGGTGTC AAAGATTCTC CTGATGTTTA TCTCACCAGC
TAGCCAACTT TTCTCCACAG TTTCTAAGAG GACTACAAAT AGAGTGGTCG

                                                                   1050
CCCCTTCCCC CAACACCTTT CCCTGATATC CACTAATAAG TGGAAGTCCT
GGGGAAGGGG GTTGTGGAAA GGGACTATAG GTGATTATTC ACCTTCAGGA

                                                                   1100
TGGTTATCAA CTCAATTTTG TTCGTAGCCT CTATTTTAAG ATAAGCCACC
ACCAATAGTT GAGTTAAAAC AAGCATCGGA GATAAAATTC TATTCGGTGG

                                                                   1150
CCGGAGGGGT GGAACTTATT GAGATTTTAG GAAAGGTAAA ATTAGCAGTT
GGCCTCCCCA CCTTGAATAA CTCTAAAATC CTTTCCATTT TAATCGTCAA

                                                                   1200
AGCTGGATTC TCATGGAAGA AATCCGATCT TAGTTCTTTG TTGCAAGGAT
TCGACCTAAG AGTACCTTCT TTAGGCTAGA ATCAAGAAAC AACGTTCCTA

                                                                   1250
TTCTAGTCTT AAAGACTTCT GTACTTCGCA ACCCCCTCCT TTGCAATTAA
AAGATCAGAA TTTCTGAAGA CATGAAGCGT TGGGGGAGGA AACGTTAATT

                                                                   1300
GCAAAGGAGG GGGGTAGAAG GAGTTTTATG AGTATATTAC TTGTTAAACC
CGTTTCCTCC CCCCATCTTC CTCAAAATAC TCATATAATG AACAATTTGG

                                                                   1350
TCCTCAACAC TAATTAGCGA CCCCTATTCC TCGGGTCGCT GTAAAATTAG
AGGAGTTGTG ATTAATCGCT GGGGATAAGG AGCCCAGCGA CATTTTAATC

                                                                   1400
TGAACAGAAG CCTTTGAAGG CTCCTTTCAA TATTAGAACA GTAATGTGGT
ACTTGTCTTC GGAAACTTCC GAGGAAAGTT ATAATCTTGT CATTACACCA

                                                                   1450
TATCACCAAT AATATGTAAG TCAATACCCG GAGACGGCTC TGGAAGATAG
ATAGTGGTTA TTATACATTC AGTTATGGGC CTCTGCCGAG ACCTTCTATC

                                                                   1500
ATTTGAGGTC TGTGGATTTT TATAAAAGAA CGTTTGATAA AATAGACAAA
TAAACTCCAG ACACCTAAAA ATATTTTCTT GCAAACTATT TTATCTGTTT
```

1550
GGAGGATTAA TTATGGTTAA AGTTATTATA CCGGACAATA ACTAATTTTA
CCTCCTAATT AATACCAATT TCAATAATAT GGCCTGTTAT TGATTAAAAT

1600
ATTTCATATT TTTCCTGTAA GTGTCGGTAA CACTTACTAG AGCCGCAAGG
TAAAGTATAA AAAGGACATT CACAGCCATT GTGAATGATC TCGGCGTTCC

1650
CGTCTATTTT ATTAAACCCC TTGTCTTTTT TAGGATCTTC AGATTTTGAG
GCAGATAAAA TAATTTGGGG AACAGAAAAA ATCCTAGAAG TCTAAAACTC

1700
TTGAAGTTAA TGAGAACTCG AAGGGGTTGA CCTATGTATT ATCGGAGTCA
AACTTCAATT ACTCTTGAGC TTCCCCAACT GGATACATAA TAGCCTCAGT

1750
TGGTGCCACA GAACGATAGG GTCCCCACAA CGAAGTTGTG GGGTCCCCCG
ACCACGGTGT CTTGCTATCC CAGGGGTGTT GCTTCAACAC CCCAGGGGGC

1800
ATTTAGTTCG AGGAATTAGA AGGGAACCAA AGGATCATAT AAGTTGCAAT
TAAATCAAGC TCCTTAATCT TCCCTTGGTT TCCTAGTATA TTCAACGTTA

1850
TTATACATTT GATGTTGAAG ATCGATGTAG AAATTGTATA TGAAATTGAG
AATATGTAAA CTACAACTTC TAGCTACATC TTTAACATAT ACTTTAACTC

1900
ACTTCGAGTA TTAGGTCCGG ATAATACTCT ACTTTCCACT CGACCCCCCC
TGAAGCTCAT AATCCAGGCC TATTATGAGA TGAAAGGTGA GCTGGGGGGG

1950
TCACCAATCT TACTTAAACT CAAATGTTGA GGATCAGCAT TAGCAACTCA
AGTGGTTAGA ATGAATTTGA GTTTACAACT CCTAGTCGTA ATCGTTGAGT

2000
TTTAAAATCC TTGTGATCCT TCTTGAAATC TTTCAAATAT TTAAAATCAT
AAATTTTAGG AACACTAGGA AGAACTTTAG AAAGTTTATA AATTTTAGTA

2050
TACTTTTATC AGATGAAAAA ATTTCTTGAT TTTGATCACT ATTTCCATTT
ATGAAAATAG TCTACTTTTT TAAAGAACTA AAACTAGTGA TAAAGGTAAA

2100
CCATTCGTTG AAAATAATCG TGCTGCAATT TTAATGTGAT ATCTGGGCAA
GGTAAGCAAC TTTTATTAGC ACGACGTTAA AATTACAGTA TAGACCCGTT

2150
ATAAAAATTA ATCATAATAT AAATATTTAC ACCCAATATC TCGATTTGGC
TATTTTTAAT TAGTATTATA TTTATAAATG TGGGTTATAG AGCTAAACCG

2200
TTTTGTGTCG AGTTTGGCGG GCCCTCATTC TATGCGTTCC TTGGGATACG
AAAACACAGC TCAAACCGCC CGGGAGTAAG ATACGCAAGG AACCCTATGC

GAATTC
CTTAAG

Table 5

Summary of Nucleotide Content of EcoR1-9 Fragments	
type I 2206 nucleotides	type II 2128 nucleotides
A - 30.4% C - 18.5% G - 19.5% T - 31.6%	A - 30.2% C - 18.7% G - 19.6% T - 31.5%
A+T - 62.0% C+G - 38.0%	A+T - 61.7% C+G - 38.3%
Repeat Region	
nucleotide 112-346 234 nucleotides (78 X 3)	nucleotide 112-268 156 nucleotides (78 X 2)
A - 35.9% C - 12.8% G - 16.7% T - 34.6%	
A+T - 70.5% C+G - 29.5%	

A computer search was made for other direct repeats as well as for inverted repeats in the EcoR1-9 fragment. The stringencies set for the direct repeat search ranged from a 50% match between bases in repeats to a 100% match. The minimum number of matched bases in the repeat sequences was set from 20 to 50 bases. No other direct sequence repeats were found outside the 78 base pair repeat region.

The stringencies used when searching for possible stem loop structures ranged again from 50% minimum percent bases matched to 100%. A summary of the potential stem loop structures in the EcoR1-9 fragment and the stringencies used are shown in table 6. None of these fall within the 78 base pair repeat region. Some fall within or just outside the potential reading frames listed in table 7 (and shown in Fig. 14), but the significance of this is not evident. Fig. 15 summarizes the location of the potential stem loop structures in the EcoR1-9 fragment. No Pst1 palindromes were found either, suggesting that there are no major mitochondrial genes as Pst1 palindromes have been noted to flank most *Neurospora* mitochondrial genes (Yin *et al.*, 1981).

A computer search was also made for possible tRNA structures in the EcoR1-9 fragment following the computer program described by Staden (1980). There were no detectable tRNA structures identified utilizing this program.

Analysis of Reading Frames in EcoR1-9

The EcoR1-9 fragments were analyzed for the presence of reading frames in both the top and bottom strands. Open reading frames greater that 50 amino acids are summarized in table 7 and Fig. 14. The position of potential start codons (Methionine codons) are also noted in both table 7 and Fig. 14.

Dr. Richard A. Collins (University of Toronto) kindly provided the sequence of the EcoR1-8 fragment which is found immediately adjacent to EcoR1-9. Analysis of the reading frames that enter the EcoR1-8 fragment revealed potential polypeptides of only 68 amino acids (top strand) and 66 amino acids (bottom strand) (Fig. 14). The sequence of the EcoR1-11 restriction fragment, which flanks the other side of EcoR1-9, was not available for analysis.

The codon usage of all the ORFs found in the EcoR1-9 fragments is summarized in table 8. Table 8 also compares these reading frames to standard and intronic mitochondrial genes in order to help determine whether these ORFs were actual reading frames that might be expressed. It has been determined that codons used in all mitochondrial genes preferentially end in A or U (80%). There are also

further biasis in the usage of certain codons which are characteristic of the standard or introhic mitochondrial genes. Codons used infrequently in standard mitochondrial genes but are found in intronic open reading frames are CGN-arg, UGG-trp, ACC-thr, ACG-thr, CUC-leu, AAG-lys and the UUU codon for phenylalanine (Nargang *et al.*, 1984). Even though these peptides are small and the frequency of codons is low, most of the potential reading frames analyzed preferentially utilize codons ending in A or U and many show a bias in the use of the mitochondrial intron codons. It can also be observed in table 7 that some of the ORFs have AUG codons (methionine) that could act as potential initiation codons as they are close to the beginning of the ORF.

The longest potential polypeptide, which is 138 amino acids long in type I mtDNA, was found on the top strand in the third reading frame and spans the repeat region. This polypeptide contains an AUG codon at amino acid position number 23 (table 7), it has 77% of its codons ending in A or U, and utilizes the UUU codon for phenylalanine exclusivlely (although there are only six occurrences of phenylalanine in this 138 amino acid polypeptide). This 138 amino acid (112 in type II mtDNA) polypeptide would be relatively hydrophilic with a polarity of 49%. Thus, this would be a soluble protein inside the mitochondria.

Table 6

Summary of Potential Stem-Loop Structures in the Type I EcoR1-9 Fragment

minper max loop	100% 2206	100% 2206	100% 2206		
min match	Nucleotides matched		nucleotides	%GC content	loop length
10	11/11		500-570 496-486	80%	4 bp
10	15/15		680-694 677-663	50%	3 bp
10	13/13		1251-1263 1244-1232	70%	7 bp
8	8/8		445-452 440-433	0%	5 bp
8	12/13		592-604 581-569	50%	23 bp
8	13/14		855-868 714-702	0%	150 bp
8	8/8		1333-1340 1323-1316	75%	10 bp
8	8/8		1345-1352 1315-1308	25%	30 bp
8	10/12		1580-1591 1574-1563	30%	6 bp
8	12/13		1735-1747 1731-1719	70%	4 bp
8	8/8		1872-1879 1863-1856	25%	9 bp
7	7/7		382-388 378-372	56%	4 bp
7	7/7		390-396 388-382	56%	2 bp
7	7/7		716-722 713-707	29%	3 bp
7	12/14		902-914 897-884	20%	5 bp

min per= minimum percent of matched bases required in stem
max loop= maximum loop allowed in strucutre
min match=minimum number of matched bases required to form stem
Nucleotides matched= ratio of nucleotides to total number of nucleotides in stem
Nucleotides= nucleotide position of stem loop structure in the EcoR1-9 type I sequence
Stem-loops shown were chosen on the basis of having over 80% of the bases in the stem-loop matched

Table 7

Summary of Potential Reading Frames over 50 Amino Acids					
Table 8 Ref Name	Reading Frame Top Strand	Nucleotides Involved	Position of Methionine	Protein Length	Percent Hydrophobicity
A	1st	514-681	Ø	56aa	33.9
B		916-1077	Ø	54aa	33.3
C	2nd	439-604	aa# 10	55aa	34.5
D		818-1036	aa# 34	73aa	31.5
E		1865-2104	aa# 74	80aa	48.8
F	3rd	35-449 (35-369)*	aa# 23	138aa (112aa)	47.1 (46.4)
G		912-1094	aa# 25	61aa	49.2
H		1581-1769	Ø	63aa	38.1
	Bottom Strand				
I	1st	1435-1638	Ø	68aa	44.1
J		1783-2118 (1783-2040)*	aa# 108	112aa 108aa	43.8
K	2nd	701-883	Ø	61aa	41.0
L	3rd	81-293	aa# 25,27, 43,65	71aa	60.6
M		294-518	aa# 36	75aa	40.0
N		1121-1343	Ø	74aa	32.4
O		1558-1745	aa# 11	69aa	31.9

* = (Type II)

Figure 14

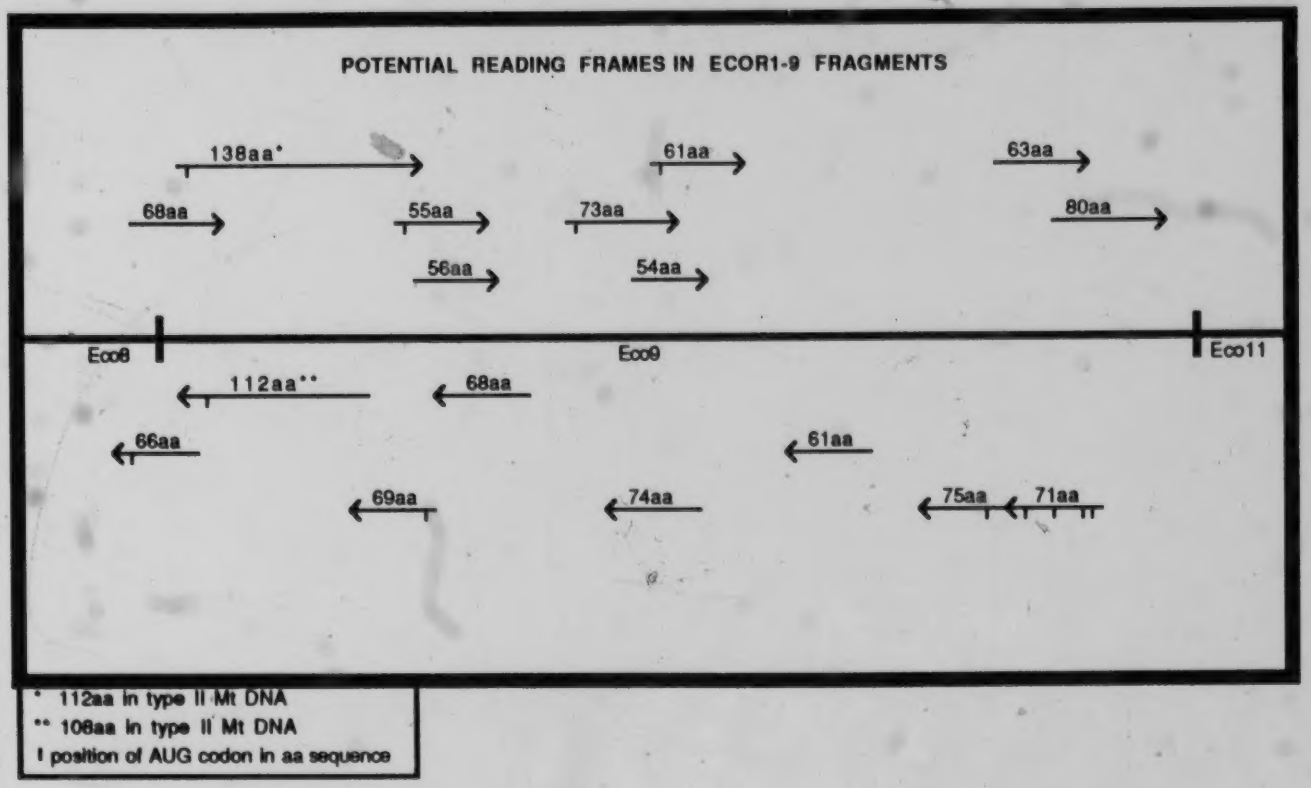
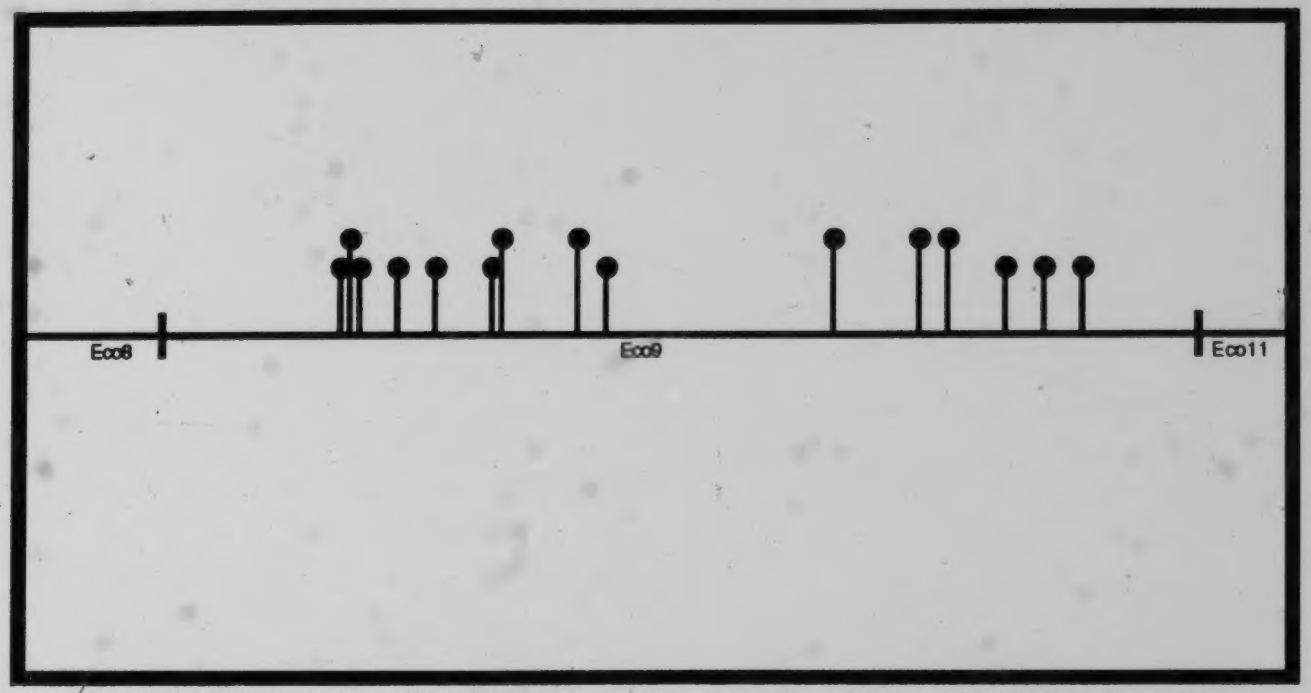

POTENTIAL READING FRAMES IN ECOR1-9 FRAGMENTS
138aa*
61aa
63aa
68aa
55aa
73aa
80aa
56aa
54aa
Eco8
Eco9
Eco11
112aa**
68aa
66aa
61aa
69aa
74aa
75aa
71aa
* 112aa in type II Mt DNA
** 108aa in type II Mt DNA
I position of AUG codon in aa sequence

Fig 15

POTENTIAL STEM LOOP STRUCTURES IN THE ECOR1-9 FRAGMENTS



Note: The hairpins indicate only the position of the stem loops and are not to scale with regard to the size of the stem or loop.

Table 8a

CODON USAGE IN POTENTIAL READING FRAMES OF TYPE1 ECOR1-9

Amino Acid	Codon	A		B		C		D		E		F*		G		H		N. CRASSA Mt genes exons**		N. CRASSA intronic ORFs**	
		Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%
ala	GCA	1	1.79	0	0.0	0	0.0	0	0.0	3	3.75	4	2.89	0	0.0	0	0.0	64	2.07	14	0.56
	GCU	0	0.0	0	0.0	1	1.81	2	2.74	1	1.25	1	0.73	0	0.0	0	0.0	84	2.72	49	1.96
	GCC	1	1.79	0	0.0	0	0.0	2	2.74	0	0.0	0	0.0	2	3.28	0	0.0	18	0.58	13	0.52
	GCG	0	0.0	0	0.0	1	1.81	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	16	0.52	9	0.36
arg	AGA	1	1.79	1	1.85	1	1.81	1	1.37	0	0.0	6	4.35	1	1.47	2	3.18	59	1.91	60	2.40
	AGG	2	3.57	1	1.85	2	3.64	1	1.37	0	0.0	1	0.73	1	1.47	2	3.18	5	0.16	17	0.68
	CGA	1	1.79	0	0.0	2	3.64	0	0.0	0	0.0	0	0.0	0	0.0	2	3.18	2	0.06	9	0.36
	CGU	1	1.79	0	0.0	0	0.0	1	1.37	1	1.25	0	0.0	0	0.0	0	0.0	4	0.13	15	0.60
	CGC	0	0.0	0	0.0	0	0.0	1	1.37	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	7	0.28
	CGG	0	0.0	0	0.0	1	1.81	1	1.37	1	1.25	0	0.0	0	0.0	0	0.0	0	0.0	3	0.12
asn	AAU	1	1.79	0	0.0	2	3.64	1	1.37	2	2.50	2	1.45	1	1.47	2	3.18	116	3.76	181	7.23
	AAC	0	0.0	1	1.85	0	0.0	1	1.37	0	0.0	5	3.62	1	1.47	1	1.59	15	0.49	34	1.36
asp	GAU	1	1.79	0	0.0	2	3.64	4	5.48	1	1.25	0	0.0	0	0.0	0	0.0	68	2.20	93	3.72
	GAC	1	1.79	0	0.0	0	0.0	1	1.37	1	1.25	0	0.0	0	0.0	0	0.0	12	0.39	11	0.44
cys	UGU	0	0.0	3	5.56	0	0.0	0	0.0	1	1.25	0	0.0	0	0.0	1	1.59	19	0.62	19	0.76
	UGC	1	1.79	0	0.0	0	0.0	1	1.37	0	0.0	0	0.0	0	0.0	1	1.59	3	0.10	1	0.04
gln	CAA	0	0.0	2	3.70	0	0.0	1	1.37	0	0.0	4	2.89	1	1.47	2	3.18	58	1.88	59	2.36
	CAG	2	3.57	1	1.85	1	1.81	1	1.37	0	0.0	4	2.89	0	0.0	0	0.0	7	0.23	7	0.28
glu	GAA	1	1.79	2	3.70	1	1.81	1	1.37	2	2.50	0	0.0	0	0.0	1	1.59	68	2.20	75	3.00
	GAG	2	3.57	0	0.0	0	0.0	1	1.37	0	0.0	4	2.90	1	1.47	3	4.76	21	0.68	23	0.92
gly	GGA	2	3.57	1	1.85	1	1.81	1	1.37	1	1.25	4	2.90	0	0.0	0	0.0	74	2.40	38	1.52
	GGU	0	0.0	0	0.0	0	0.0	5	6.85	0	0.0	4	2.90	2	3.28	0	0.0	105	3.40	63	2.52
	GGC	0	0.0	2	3.70	1	1.81	0	0.0	1	1.25	0	0.0	0	0.0	1	1.59	5	0.16	5	0.20
	GGG	2	3.57	0	0.0	1	1.81	1	1.37	0	0.0	0	0.0	0	0.0	3	4.76	26	0.84	20	0.80
his	CAU	1	1.79	1	1.85	1	1.81	0	0.0	1	1.25	3	2.17	0	0.0	0	0.0	52	1.68	32	1.28
	CAC	0	0.0	1	1.85	0	0.0	2	2.74	0	0.0	1	0.73	1	1.47	2	3.18	14	0.45	4	0.16
ile	AUU	1	1.79	0	0.0	0	0.0	6	8.23	2	2.50	6	4.35	4	6.56	1	1.59	134	4.34	98	3.92
	AUA	3	5.36	1	1.85	0	0.0	0	0.0	2	2.50	11	7.97	2	3.28	1	1.59	172	5.57	124	4.95
	AUC	0	0.0	0	0.0	1	1.81	1	1.37	1	1.25	3	2.17	3	4.92	1	1.59	40	1.30	10	0.40

leu	UUA	0	0.0	3	5.56	1	1.81	0	0.0	7	8.75	6	4.35	1	1.47	2	3.18	300	9.71	178	7.11
	UUG	0	0.0	0	0.0	2	3.64	3	4.11	2	2.50	3	2.17	3	4.92	1	1.59	43	1.39	22	0.88
	CUA	2	3.57	1	1.85	0	0.0	2	2.74	1	1.25	4	2.90	0	0.0	1	1.59	52	1.68	36	1.44
	CUU	0	0.0	0	0.0	1	1.81	1	1.37	2	2.50	3	2.17	2	3.28	1	1.59	50	1.62	41	1.64
	CUC	1	1.79	1	1.85	2	3.64	1	1.37	3	3.75	2	1.45	1	1.47	0	0.0	4	0.13	7	0.28
	CUG	1	1.79	0	0.0	3	5.46	0	0.0	1	1.25	0	0.0	2	3.28	0	0.0	4	0.13	7	0.28
lys	AAA	3	5.36	0	0.0	3	5.46	2	2.74	8	10.0	4	2.90	1	1.47	1	1.59	66	2.14	199	7.95
	AAG	3	5.36	1	1.85	1	1.81	0	0.0	0	0.0	0	0.0	3	4.92	1	1.59	9	0.29	59	2.36
met	AUG	0	0.0	0	0.0	0	0.0	1	1.37	1	1.25	1	0.73	1	1.47	0	0.0	79	2.56	34	1.36
phe	UUU	1	1.79	3	5.56	2	5.46	1	1.37	4	5.00	6	4.35	1	1.47	5	7.94	184	5.96	119	4.75
	UUC	1	1.79	1	1.85	1	1.81	1	1.37	3	3.75	0	0.0	2	3.28	1	1.59	76	2.46	24	0.96
pro	CCA	1	1.79	3	5.56	1	1.81	1	1.37	4	5.00	0	0.0	2	3.28	1	1.59	26	0.84	22	0.88
	CCU	2	3.57	2	3.70	1	1.81	4	5.48	0	0.0	0	0.0	0	0.0	0	0.0	73	2.36	49	1.96
	CCC	2	3.57	3	5.56	2	5.46	2	2.74	2	2.50	1	0.73	3	4.92	2	3.18	4	0.13	8	0.32
	CCG	0	0.0	0	0.0	1	1.81	1	1.37	0	0.0	0	0.0	0	0.0	1	1.59	5	0.16	2	0.08
ser	UCA	1	1.79	1	1.85	0	0.0	0	0.0	5	6.25	1	0.73	3	4.92	1	1.59	56	1.81	49	1.96
	UCU	1	1.79	1	1.85	0	0.0	2	2.74	2	2.50	4	2.90	1	1.47	0	0.0	95	3.08	73	2.92
	UCC	0	0.0	2	3.70	1	1.81	1	1.37	2	2.50	2	1.45	3	4.92	1	1.59	13	0.42	18	0.72
	UCG	0	0.0	2	3.70	0	0.0	1	1.37	0	0.0	0	0.0	0	0.0	3	4.76	5	0.16	13	0.52
	AGU	1	1.79	2	3.70	2	5.46	0	0.0	0	0.0	5	3.62	0	0.0	2	3.18	103	3.33	53	2.12
	AGC	0	0.0	0	0.0	1	1.81	1	1.37	0	0.0	0	0.0	0	0.0	0	0.0	19	0.62	11	0.44
thr	ACA	0	0.0	0	0.0	0	0.0	1	1.37	0	0.0	8	5.80	0	0.0	0	0.0	77	2.49	31	1.24
	ACU	0	0.0	1	1.85	1	1.81	1	1.37	1	1.25	3	2.17	2	3.28	0	0.0	70	2.27	59	2.36
	ACC	0	0.0	1	1.85	0	0.0	2	2.74	0	0.0	1	0.73	1	1.47	1	1.59	8	0.26	9	0.36
	ACG	2	3.57	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.59	3	0.10	8	0.32
trp	UGA	0	0.0	2	3.70	1	1.81	0	0.0	5	6.25	1	0.73	2	3.28	1	1.59	49	1.59	26	1.04
	UGG	0	0.0	2	3.70	1	1.81	0	0.0	0	0.0	1	0.73	2	3.28	1	1.59	3	0.10	6	0.24
tyr	UAU	2	3.57	2	3.70	0	0.0	4	5.48	2	2.50	5	3.62	0	0.0	2	3.18	111	3.59	102	4.08
	UAC	1	1.79	0	0.0	0	0.0	0	0.0	1	1.25	2	1.45	0	0.0	0	0.0	35	1.13	17	0.68
val	GUA	2	3.57	1	1.85	1	1.81	0	0.0	0	0.0	5	3.62	2	3.28	1	1.59	84	2.72	54	2.16
	GUU	0	0.0	1	1.85	1	1.81	1	1.37	1	1.25	5	3.62	1	1.47	2	3.18	87	2.82	56	2.24
	GUC	0	0.0	0	0.0	1	1.81	1	1.37	1	1.25	0	0.0	0	0.0	3	4.76	10	0.32	8	0.32
	GUG	0	0.0	0	0.0	1	1.81	0	0.0	0	0.0	1	0.73	1	1.47	0	0.0	25	0.81	10	0.40
%G or C		22/56		19/54		25/55		28/73		14/80		32/138		31/61		30/60					
n 3rd Position		39%		35%		71%		38%		17%		23%		50%		47%		17%		17%	

* 138 aa polypeptide that spans the repeat region ** Taken from S. Pande's PhD Thesis, University of Alberta, 1988

Note: Reading Frames analyzed correspond to Reading Frames of Table 7

Table 8b

CODON USAGE IN POTENTIAL READING FRAMES OF TYPE I ECORI-9

Amino Acid	Codon	I		J		K		L		M		N		O		N. CRASSA Mt genes exons**		N. CRASSA intronic ORFs**	
		Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%	Frq	%
ala	GCA	1	1.47	3	2.68	0	0.0	0	0.0	1	1.33	0	0.0	0	0.0	64	2.07	14	0.56
	GCU	3	4.41	2	1.79	1	1.64	0	0.0	0	0.0	0	0.0	1	1.45	84	2.72	49	1.96
	GCC	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.45	18	0.58	13	0.52
	GCG	0	0.0	0	0.0	1	1.81	0	0.0	0	0.0	0	0.0	0	0.0	16	0.52	9	0.36
arg	AGA	1	1.47	3	2.68	1	1.64	1	1.41	2	2.67	1	1.35	0	0.0	59	1.91	60	2.40
	AGG	1	1.47	1	0.89	0	0.0	2	2.82	1	1.33	0	0.0	1	1.45	5	0.16	17	0.68
	CGA	0	0.0	0	0.0	1	1.64	1	1.41	4	5.33	0	0.0	0	0.0	2	0.06	9	0.36
	CGU	0	0.0	0	0.0	1	1.64	0	0.0	1	1.33	1	1.35	1	1.45	4	0.13	15	0.60
	CGC	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.45	0	0.0	7	0.28
	CGG	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.45	0	0.0	3	0.12
asn	AAU	1	1.47	6	5.36	0	0.0	2	2.82	3	4.00	4	5.41	0	0.0	116	3.76	181	7.23
	AAC	2	2.94	3	2.68	1	1.64	1	1.41	0	0.0	1	1.35	1	1.45	15	0.49	34	1.36
asp	GAU	3	4.41	0	0.0	1	1.64	2	2.82	1	1.33	4	5.41	1	1.45	68	2.20	93	3.72
	GAC	0	0.0	1	0.89	1	1.64	0	0.0	1	1.33	0	0.0	1	1.45	12	0.39	11	0.44
cys	UGU	1	1.47	5	4.46	1	1.64	0	0.0	0	0.0	0	0.0	0	0.0	19	0.62	19	0.76
	UGC	0	0.0	2	1.79	0	0.0	1	1.41	0	0.0	0	0.0	1	1.45	3	0.10	1	0.04
gln	CAA	0	0.0	1	0.89	1	1.64	1	1.41	3	4.00	4	5.41	2	2.90	58	1.88	59	2.36
	CAG	1	1.47	0	0.0	1	1.64	1	1.41	0	0.0	2	2.70	2	2.90	7	0.23	7	0.28
glu	GAA	0	0.0	1	0.89	1	1.64	2	2.82	1	1.33	3	4.05	1	1.45	68	2.20	75	3.00
	GAG	0	0.0	3	2.68	2	3.28	1	1.41	0	0.0	2	2.70	2	2.90	21	0.68	23	0.92
gly	GGA	3	4.41	0	0.0	1	1.64	0	0.0	2	2.67	3	4.05	1	1.45	74	2.40	38	1.52
	GGU	1	1.47	0	0.0	2	3.28	0	0.0	2	2.67	1	1.35	0	0.0	105	3.40	63	2.52
	GGC	1	1.47	0	0.0	1	1.64	0	0.0	0	0.0	2	2.70	1	1.45	5	0.16	5	0.20
	GGG	1	1.47	1	0.89	0	0.0	0	0.0	2	2.67	3	4.05	4	5.80	26	0.84	20	0.80
his	CAU	1	1.47	0	0.0	1	1.64	1	1.41	1	1.33	1	1.35	0	0.0	52	1.68	32	1.28
	CAC	0	0.0	1	0.89	2	3.28	1	1.41	0	0.0	1	1.35	1	1.45	14	0.45	4	0.16
ile	AUU	2	2.94	13	11.61	5	8.20	7	9.86	1	2.33	2	2.70	2	2.90	134	4.34	98	3.92
	AUA	0	0.0	2	1.79	2	3.28	4	5.63	2	2.67	0	0.0	0	0.0	172	5.57	124	4.95
	AUC	2	2.94	0	0.0	3	4.92	5	7.04	1	1.33	0	0.0	2	2.90	40	1.30	10	0.40

leu	UUA	1	1.47	3	2.68	2	3.28	5	7.04	1	1.33	0	0.0	1	1.45	300	9.71	178	7.11
	UUG	1	1.47	0	0.0	1	1.64	2	2.82	3	4.00	1	1.35	0	0.0	43	1.39	22	0.88
	CUA	3	4.41	6	5.36	2	3.28	1	1.41	1	1.33	2	2.70	1	1.45	52	1.68	36	1.44
	CUU	1	1.47	5	4.46	1	1.64	0	0.0	3	4.00	3	4.05	2	2.70	50	1.62	41	1.64
	CUC	2	2.94	0	0.0	0	0.0	1	1.41	2	2.67	2	2.70	1	1.45	4	0.13	7	0.28
	CUG	2	2.94	0	0.0	0	0.0	2	2.82	0	0.0	0	0.0	3	4.35	4	0.13	7	0.28
lys	AAA	2	2.94	6	5.36	4	6.56	4	5.63	2	2.67	3	4.05	1	1.45	66	2.14	199	7.95
	AAG	2	2.94	0	0.0	0	0.0	2	2.82	0	0.0	2	2.70	1	1.45	9	0.29	59	2.36
met	AUG	0	0.0	1	0.89	0	0.0	4	5.63	1	1.33	0	0.0	1	1.45	79	2.56	34	1.36
phe	UUU	3	4.41	1	0.89	3	4.92	5	7.04	1	1.33	2	2.70	4	5.80	184	5.96	119	4.75
	UUC	1	1.47	0	0.0	2	3.28	0	0.0	2	2.67	0	0.0	0	0.0	76	2.46	24	0.96
pro	CCA	0	0.0	0	0.0	1	1.64	1	1.41	1	1.33	2	2.79	1	1.45	26	0.84	22	0.88
	CCU	2	2.94	7	6.25	1	1.64	0	0.0	2	2.67	2	2.70	3	4.35	73	2.36	49	1.96
	CCC	0	0.0	0	0.0	1	1.64	0	0.0	2	2.67	0	0.0	2	2.90	4	0.13	8	0.32
	CCG	1	1.47	0	0.0	2	3.28	0	0.0	0	0.0	0	0.0	1	1.45	5	0.16	2	0.08
ser	UCA	1	1.47	0	0.0	1	1.64	1	1.41	0	0.0	2	2.70	1	1.45	56	1.81	49	1.96
	UCU	1	1.47	0	0.0	2	3.28	0	0.0	1	1.33	0	0.0	1	1.45	95	3.08	73	2.92
	UCC	3	4.41	3	2.68	2	3.28	0	0.0	3	4.00	0	0.0	1	1.45	13	0.42	18	0.72
	UCG	0	0.0	0	0.0	0	0.0	0	0.0	2	2.67	1	1.47	3	4.35	5	0.16	13	0.52
	AGU	0	0.0	0	0.0	0	0.0	0	0.0	2	2.67	2	2.70	1	1.45	103	3.33	53	2.12
	AGC	0	0.0	3	2.68	0	0.0	0	0.0	0	0.0	0	0.0	1	1.45	19	0.62	11	0.44
thr	ACA	0	0.0	8	7.14	0	0.0	2	2.82	1	1.33	0	0.0	0	0.0	77	2.49	31	1.24
	ACU	2	2.94	2	1.79	1	1.64	0	0.0	3	4.00	0	0.0	3	4.35	70	2.27	59	2.36
	ACC	1	1.47	0	0.0	0	0.0	0	0.0	0	0.0	2	2.70	0	0.0	8	0.26	9	0.36
	ACG	1	1.47	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.45	3	0.10	8	0.32
trp	UGA	1	1.47	3	2.68	0	0.0	3	4.23	2	2.67	3	4.05	1	1.45	49	1.59	26	1.04
	UGG	1	1.47	0	0.0	0	0.0	0	0.0	1	1.33	2	2.70	1	1.45	3	0.10	6	0.24
tyr	UAU	2	2.94	3	2.68	2	3.28	1	1.41	2	2.67	2	2.70	1	1.45	111	3.59	102	4.08
	UAC	3	4.41	0	0.0	0	0.0	0	0.0	2	2.67	3	4.05	1	1.45	35	1.13	17	0.68
val	GUA	4	5.88	4	3.57	0	0.0	1	1.41	2	2.67	0	0.0	0	0.0	84	2.72	54	2.16
	GUU	1	1.47	6	5.36	1	1.64	1	1.41	0	0.0	1	1.35	0	0.0	87	2.82	56	2.24
	GUC	0	0.0	0	0.0	1	1.64	0	0.0	0	0.0	0	0.0	1	1.45	10	0.32	8	0.32
	GUG	0	0.0	2	1.79	0	0.0	1	1.41	3	4.00	1	1.35	0	0.0	25	0.81	10	0.40
%G or C in 3rd Position		26/71 37%		21/75 28%		20/74 27%		24/69 35%		26/61 43%		25/68 37%		37/112 33%		17%		17%	

** Taken from S. Pande's PhD Thesis, University of Alberta, 1988

Note: Reading Frames analyzed correspond to Reading Frames of Table 7

Heterokaryon Study

Heterokaryons were constructed by superimposing conidia from NCN 10 and NCN 45 on minimal medium as described in Materials and Methods. Figures 16 a) to 16 g) show EcoR1 digests of the mtDNA of heterokaryons that were subcultured for several generations. On these gels the conversion of the EcoR1-5 and EcoR1-9 fragments can be monitored through the generations of subculturing. Table 9 provides a summary of the unidirectional conversion involving the EcoR1-9 fragments in the 20 heterokaryons and also the suppressivity of the [*mi-3*] phenotype (as determined by cytochrome spectral analysis) over wildtype. Cytochrome spectra of NCN 10 (wildtype) and NCN 45 ([*mi-3*]) and three heterokaryons representing a wildtype, "mid" and [*mi-3*] heterokaryons are shown in Fig 17. It appears that [*mi-3*] is not completely suppressive as only 7 of the 20 heterokaryons had become [*mi-3*] by the ninth passage of conidia. The EcoR1-5 fragment converted 100% to the type II (insert containing form) by the second conidial passage in each of the 5 heterokaryons analyzed (indicated by + in table 9). The EcoR1-9 fragment converted to the type II form in 70% of the cases, as was expected from previous reports (Lemire and Nargang, 1980), although the resolution took much longer than the EcoR1-5 fragment conversion. Interestingly, among all the [*mi-3*] suppressive heterokaryons, those that resolved to contain the type I EcoR1-9 fragment appear to resolve at a faster rate than the rest. In fact, all 6 heterokaryons that resolved to type I resolved much faster than those that resolved (or were in the process of resolution) to type II EcoR1-9. The resolution to the type II EcoR1-9 fragment in the two heterokaryons that became [*mi-3*] appeared to occur quicker than the conversion in the heterokaryons that became wildtype. These observations may be a reflection of the take over of the [*mi-3*] mitochondria in these heterokaryons. Those heterokaryons that show the wildtype phenotype may still have a mixture of mitochondria since wildtype appears to be dominant to [*mi-3*]. Thus, one would also expect to see a mixture of type I and type II mtDNAin the heterokaryons displaying the wildtype phenotype.

The resolution bias observed for the EcoR1-9 polymorphic marker in the heterokaryon studies by Mannella and Lambowitz(1979) and Lemire and Nargang(1986) were not examined by χ^2 analysis. Table 10 provides the results of a χ^2 test on the resolution of the type I and type II EcoR1-9 fragments in Mannella

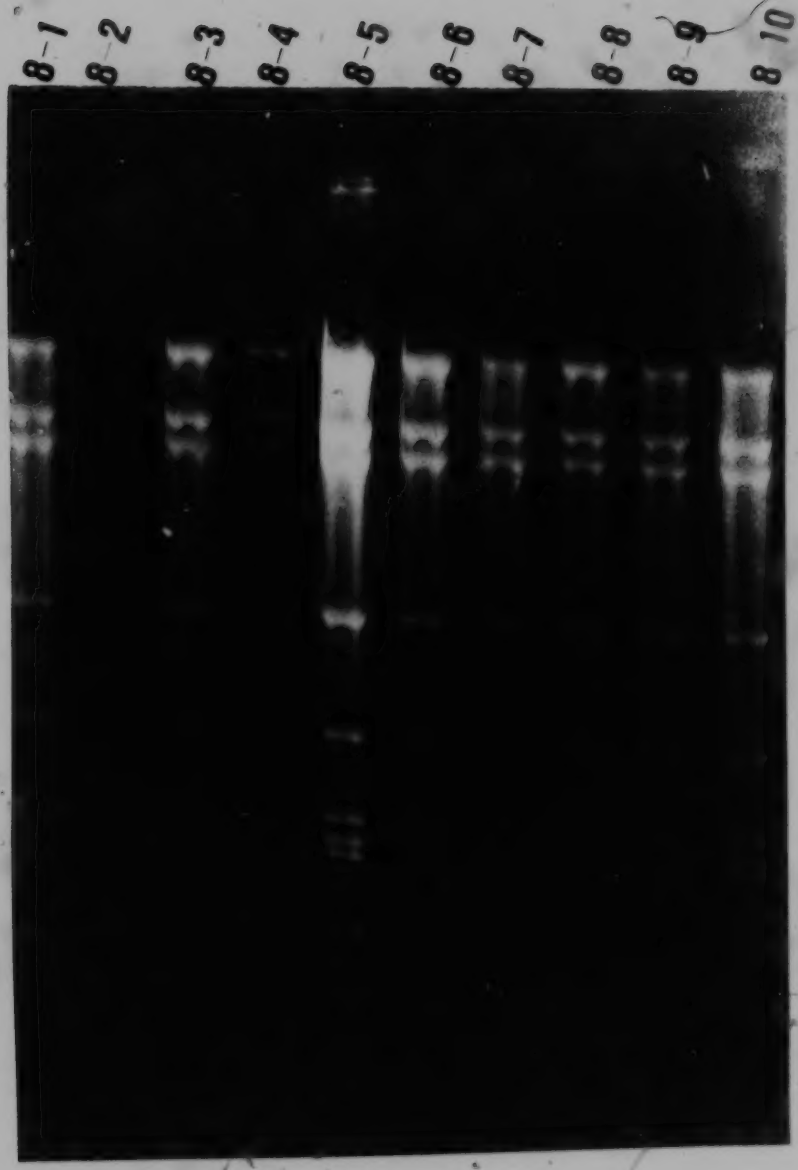
and Lambowitz's [*poky*]/wildtype heterokaryon study and for pooled data from the [*mi-3*]/ wildtype heterokaryon studies of this study and those of Lemire and Nargang(1986). The null hypothesis assumed a 1:1 resolution of the two polymorphic forms in each case. The results for the [*poky*]/wildtype heterokaryons suggests that the null hypothesis should be rejected since with one degree of freedom the probability of obtaining the observed ratio is less than 0.01. Similarly, with one degree of freedom the probability of resolving the EcoR1-9 fragments in the observed ration in the [*mi-3*]/wildtype heterokaryons is also low (ca. 0.025). Thus, in both cases, the resolution of the EcoR1-9 fragments does not appear to follow a simple 1:1 relationship. Since the χ^2 test supports the hypothesis that a simple reciprocal recombination mechanism does not account for the data, the resolution of EcoR1-9 fragments will be referred to as "conversion" to maintain consistency with the literature. (see Mannella and Lambowitz,1979)

Figure 16 a) to g)

Agarose Gels of Heterokaryon Mitochondrial DNA Digested With EcoR1

Figures 16 a) to g) are pictures of Heterokaryon mitochondrial DNA digested with EcoR1 and run on 0.8% agarose gels. The first digit of each lane refers to the heterokaryon generation, the second refers to the heterokaryon isolate number. NCN 20 and NCN 10 mitochondrial DNA digested with EcoR1 were the type I and type II markers DNA, respectively. The first mitochondrial DNA isolations were performed on the eighth generation heterokaryons. Depending on the characteristics found in the eighth generation, isolation of mtDNA from either previous or subsequent generations was done to determine the generation at which the EcoR1-9 fragments resolved to the type I or type II polymorphic form.

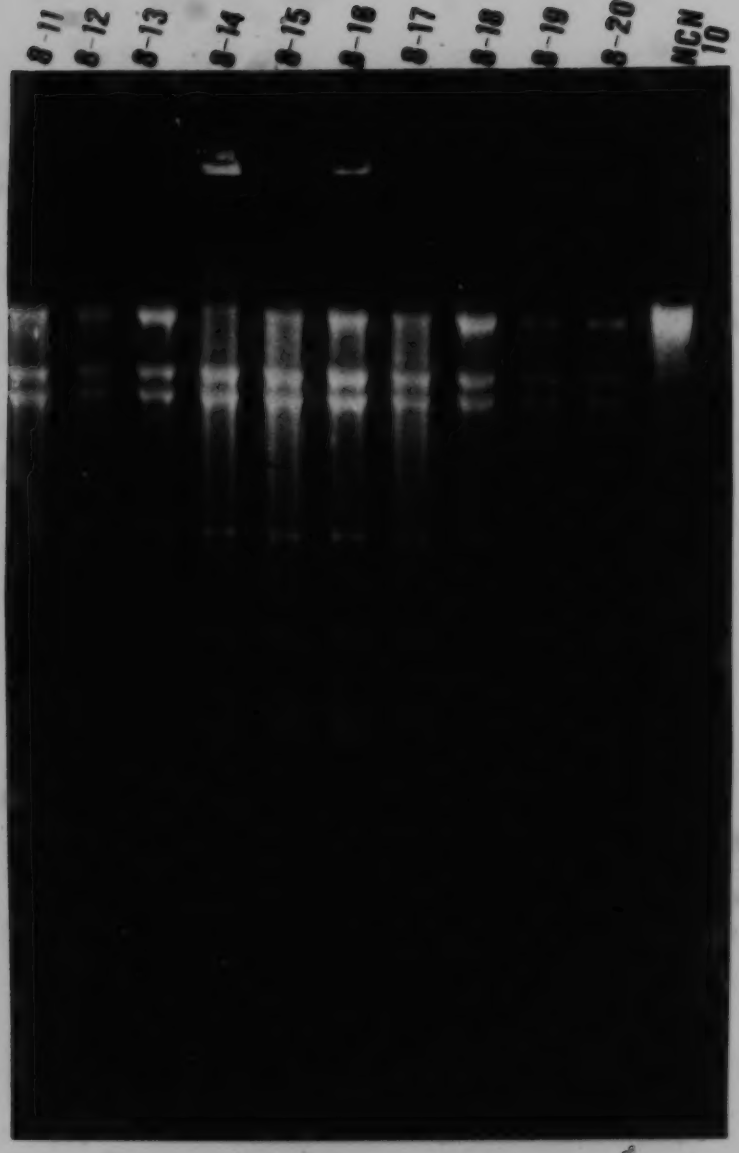
a



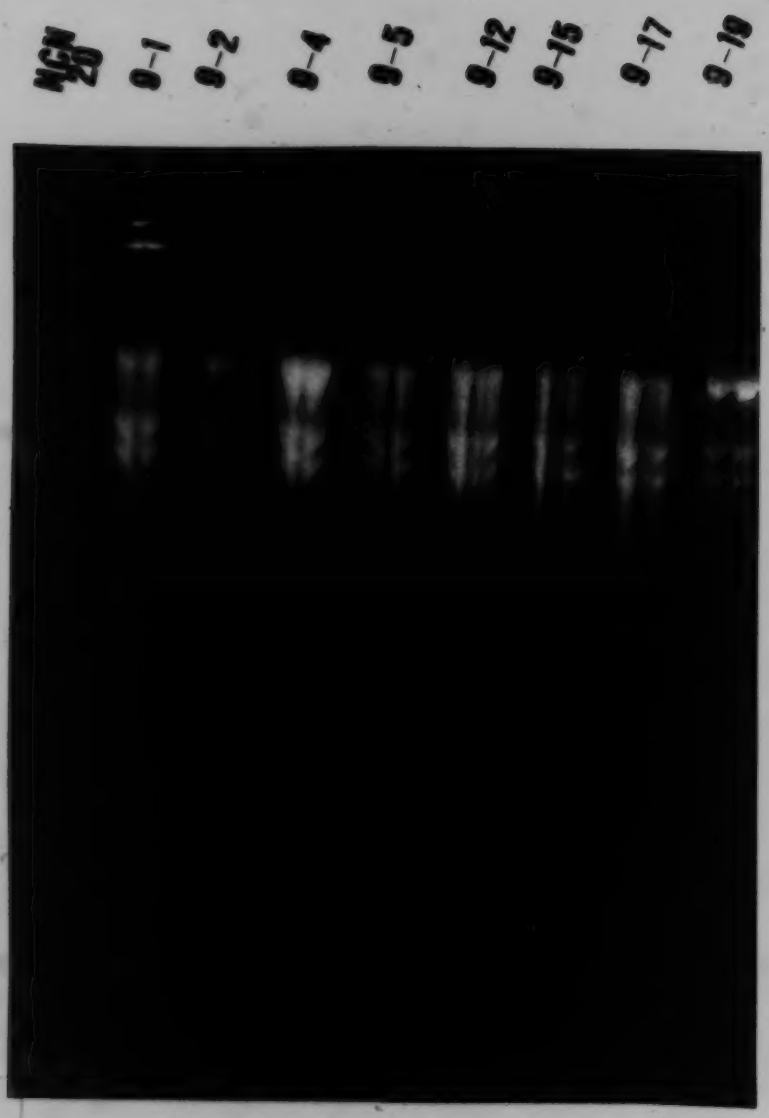
9(I)

9(II)

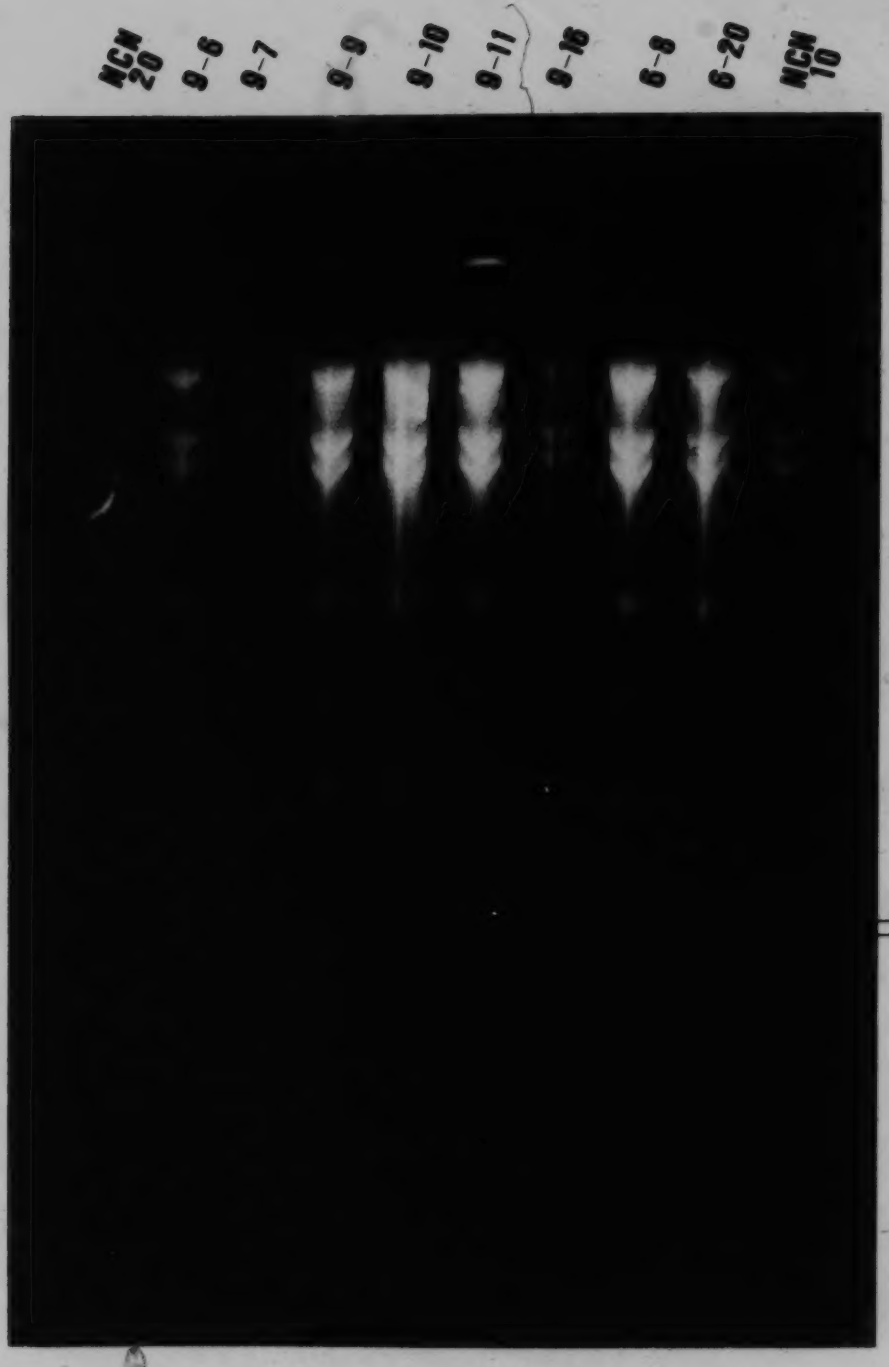
b



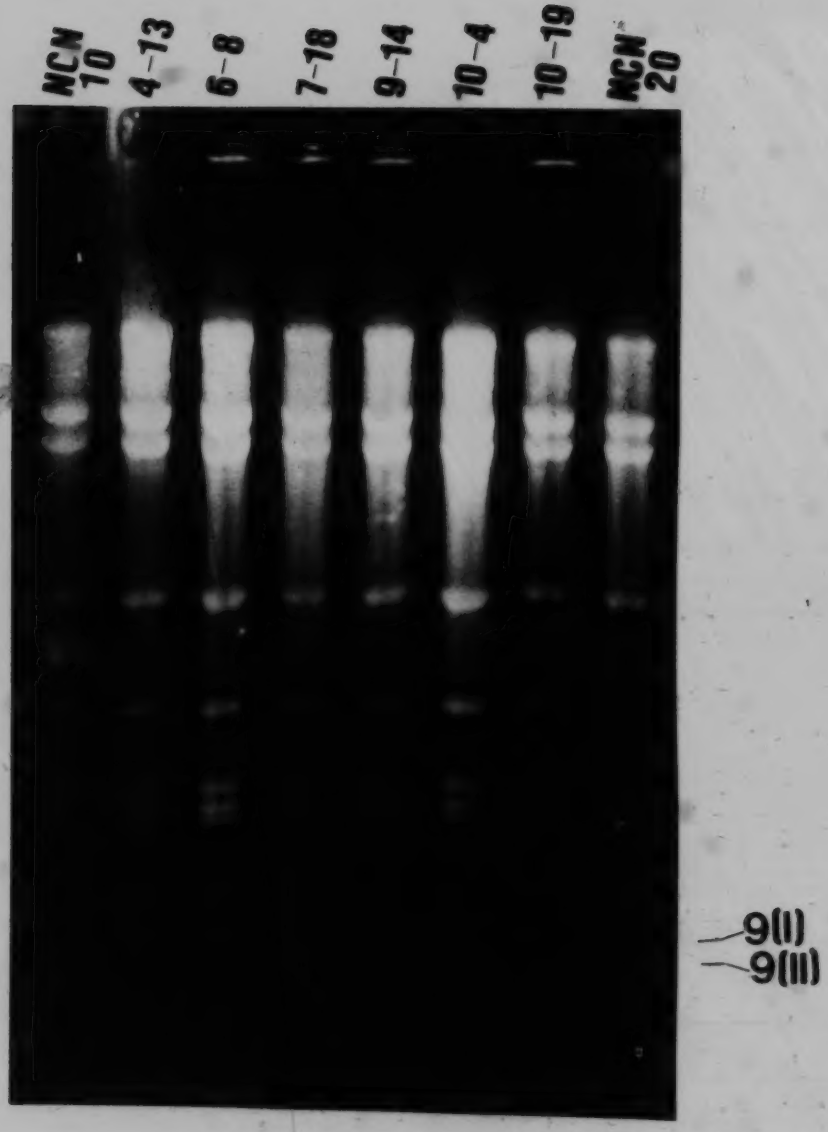
c



d



e


NCN 10
4-13
6-8
7-18
9-14
10-4
10-19
NCN 20
9(I)
9(II)

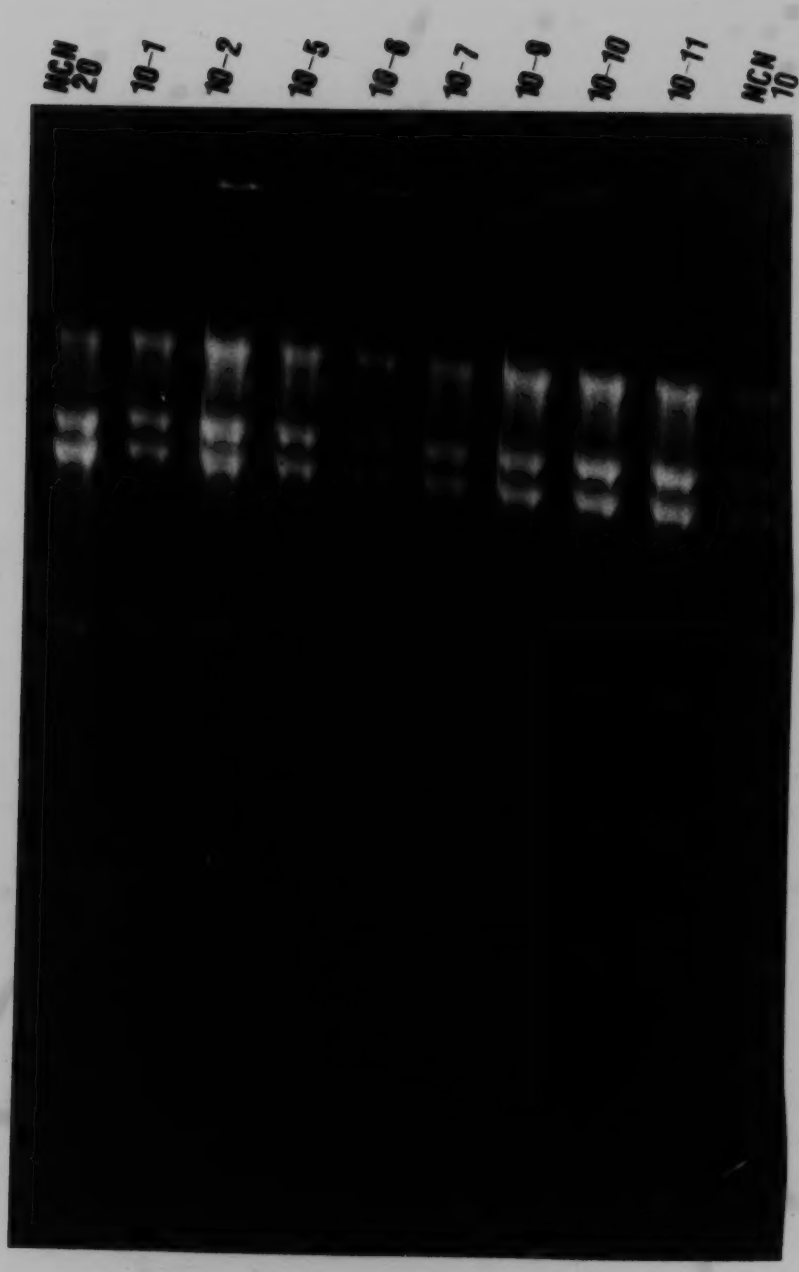

NCN 20
10-1
10-2
10-5
10-6
10-7
10-9
10-10
10-11
NCN 10
9(I)
9(II)

Table 9

Summary of Resolution of the EcoR1-9 Fragments and Suppressivity of [Mt-3] in Heterokaryons Between [Mt-3] and Wildtype Strains of Neurospora crassa

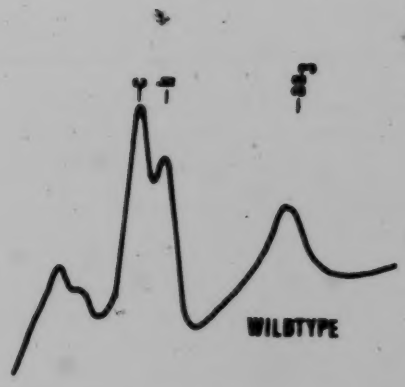
Heterokaryon																				
	1		2		3		4		5		6		7		8		9		10	
Heterokaryon generation	DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type	
1	-	-	-	-	-	-	-	wt	-	-	-	-	-	-	-	-	-	-	-	-
2	mix	wt	-	wt	-	-	mix	wt	-	-	-	-	-	-	-	mid	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	mix	mid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	mix	wt	I	mt-3	-	-	-	-	-	-	-	-	I	mt-3	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	mix	wt	mix	wt	I	mt-3	mix	mid	mix	wt	mix	mid	mix	wt	I	mt-3	mix	wt	mix	wt
9	mix	wt	mix	wt	I	mt-3	mix	mt-3	mix	wt	mix	mt-3	mix	wt	I	mt-3	mix	wt	mix	wt
10	II	wt	II	wt	-	-	II	mt-3	II	wt	II	mt-3	II	wt	-	-	II	wt	II	wt
	11		12		13		14		15		16		17		18		19		20	
Heterokaryon generation	DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type		DNA Spectra Type	
1	-	-	-	-	-	-	-	wt	-	-	-	wt	-	-	-	-	-	-	I**	mt-3
2	-	-	-	-	mix	wt	-	-	-	-	-	-	mix	wt	-	mt-3	-	-	I	mt-3
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	I	mt-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	I	mt-3	mix	wt	-	-	-	-	-	-	mix°	wt	-	-	I	mt-3
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	mix°	wt	-	-	-	-
8	mix	wt	mix	wt	I	mt-3	mix	wt	mix	wt	mix	wt	mix	wt	I	wt	mix°	mid	I	mt-3
9	mix	wt	mix	wt	-	-	II	wt	mix	wt	II	wt	mix	wt	-	-	I	mt-3	-	-
10	II	wt	II	wt	-	-	-	-	II	wt	II	wt	II	wt	-	-	I	mt-3	-	-

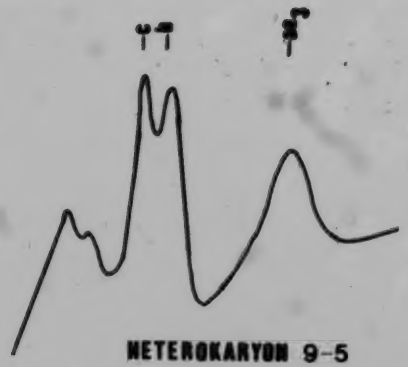
Note: "mix refers only to the EcoR1-9 fragment. All mtDNA analysed was resolved to the type II EcoR1-5 fragment except where noted by **.
Except where noted by °, mixtures of EcoR1-9's had a larger proportion of Type II EcoR1-9 fragment.
** Mixture of EcoR1-5 fragments but not of EcoR1-9 fragments.
In some cases DNA recovery was insufficient to determine resolution of the EcoR1-9 fragments.

Figure 17

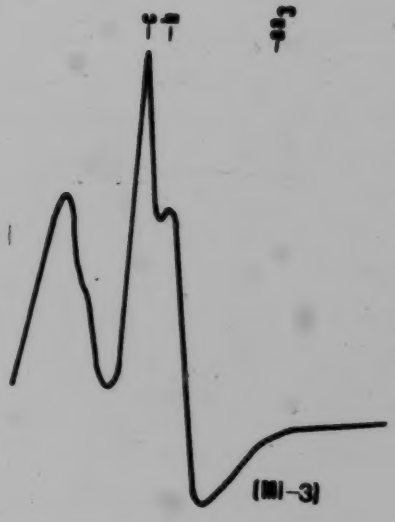
Cytochrome Spectra of Heterokaryons

Cytochrome spectra of NCN 10 (wildtype) NCN 45 ([*mi-3*]) and three heterokaryons. Heterokaryon 9-5 represents a wildtype heterokaryon and 10-4 represents a heterokaryon with an [*mi-3*] phenotype. Heterokaryon 8-6 represents a mid phenotype heterokaryon because it has a small amount of cytochrome aa_3.


WILDTYPE


HETEROKARYON 9-5

HETEROKARYON 8-6


(MI-3)

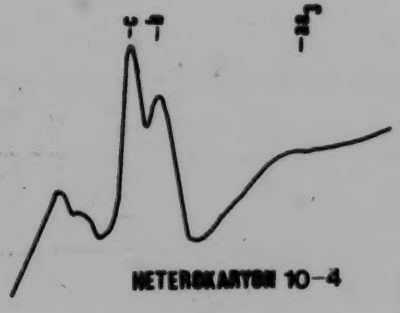

HETEROKARYON 10-4

Table 10

Chi Squared Test

Heterokaryons	Total Number of Heterokaryons Analyzed	EcoR1-9 Resolved to type I	$\frac{(Obs - Exp)^2}{Exp}$	EcoR1-9 Resolved to type II	$\frac{(Obs - Exp)^2}{Exp}$	Chi Square Value	P
[Poky]/wildtype*	34	25	3.75	9	3.75	7.5	>0.01
[mi-3]/wildtype**	30	9	2.4	21	2.4	4.8	~ 0.025

* Results taken from Mannella and Lambowitz (1979)

**Results pooled from present study and Lemire and Nargang (1986)

Cloning and Sequence Analysis of the Type I and Type II EcoR1-5 Fragments

The DNA sequence of the *Neurospora crassa* EcoR1-5, type II, mitochondrial DNA fragment has recently been determined (Werner and Burger, 1985). The fragment was found to contain a long unidentified reading frame (URF) named URF1 which is now known to encode a component of the NADH dehydrogenase complex (Chomyn et al., 1985). URF 1 was found to be interrupted by a single intron (Fig. 17a). This intron within URF1 contained another open reading frame. In the present study, fragments from both the type I and type II EcoR1-5 fragments spanning the site of insertion of the intron were cloned into M13mp18. DNA sequence analysis revealed that the URF1 intron corresponds exactly to the ca. 1200 base pair insertion found in the type II EcoR1-5 fragment (Mannella and Lambowitz, 1978; Mannella and Lambowitz, 1979) and is excised precisely from the type I polymorphic form of EcoR1-5. The type I EcoR1-5 sequence confirms the predicted splice sites of Burger and Werner (1985). (Fig. 17a and 17b)

Analysis of the URF1 Intron

The conversion of the *Neurospora* URF1 intron of the type II EcoR1-5 fragment in heterokaryons resembles the unidirectional conversion of omega in the intron of the 21S rRNA gene of yeast. The omega gene has been shown to encode a double strand endonuclease responsible for the conversion of the 21S rRNA gene to the intron containing form (Jaquier and Dujon, 1985; Zinn and Butow, 1985; Zinn and Butow, 1986). Comparison of the ORF found in the URF1 intron to the omega protein revealed no amino acid homology between the two. An amino acid computer search of the microgenie computer banks revealed no sequence homologies to any proteins in the computer data bank. It was thought that the *Neurospora crassa* URF1 intron may encode an excision protein or a protein involved in conversion of type I EcoR1-5 to type II. (see fig. 18) Sequence homology to the *Podospora anserina* URF1 intronic ORFs was found (Burger and Werner, 1985) but there has been no function attributed to these potential proteins.

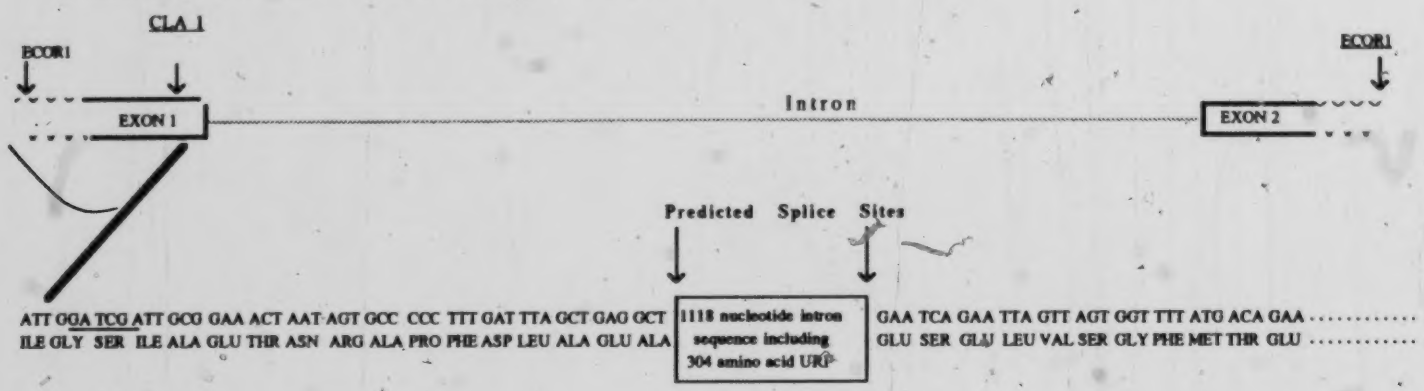
Figure 18

DNA Sequence of Cla1/EcoR1 Fragment From the EcoR1-5 Fragments of Both Type I and Type II *N. crassa* Mitochondrial DNA

A Cla1/EcoR1 fragment (that covers the URF1 intron) from both type I and type II EcoR1-5 was cloned and sequenced. The URF1 intron corresponds exactly to the 1118 bp insertion found in the type II EcoR1-5 and is excised precisely from the type I polymorphic form of EcoR1-5 (Fig 16b). The type I EcoR1-5 sequence confirms the predicted splice sites of Burger and Werner (1985).

Figure 18

a) EcoR1-5 Type II Cla 1/EcoR1 Fragment Sequence


CLA 1
ECOR1
ECOR1
EXON 1
Intron
EXON 2
Predicted Splice Sites
ATT GGA TCG ATT GCG GAA ACT AAT AGT GCC CCC TTT GAT TTA GCT GAG GCT
ILE GLY SER ILE ALA GLU THR ASN ARG ALA PRO PHE ASP LEU ALA GLU ALA
1118 nucleotide intron sequence including 304 amino acid URF
GAA TCA GAA TTA GTT AGT GGT TTT ATG ACA GAA .....
GLU SER GLU LEU VAL SER GLY PHE MET THR GLU .....

b) EcoR1-5 Type I Cla 1/ EcoR1 Fragment Sequence

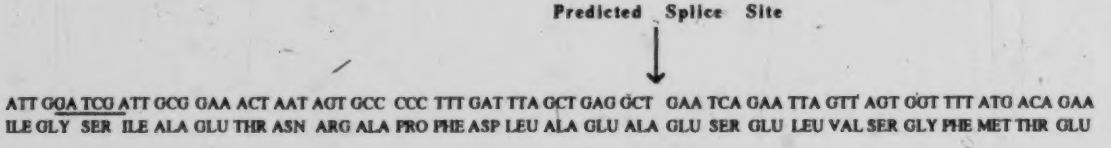
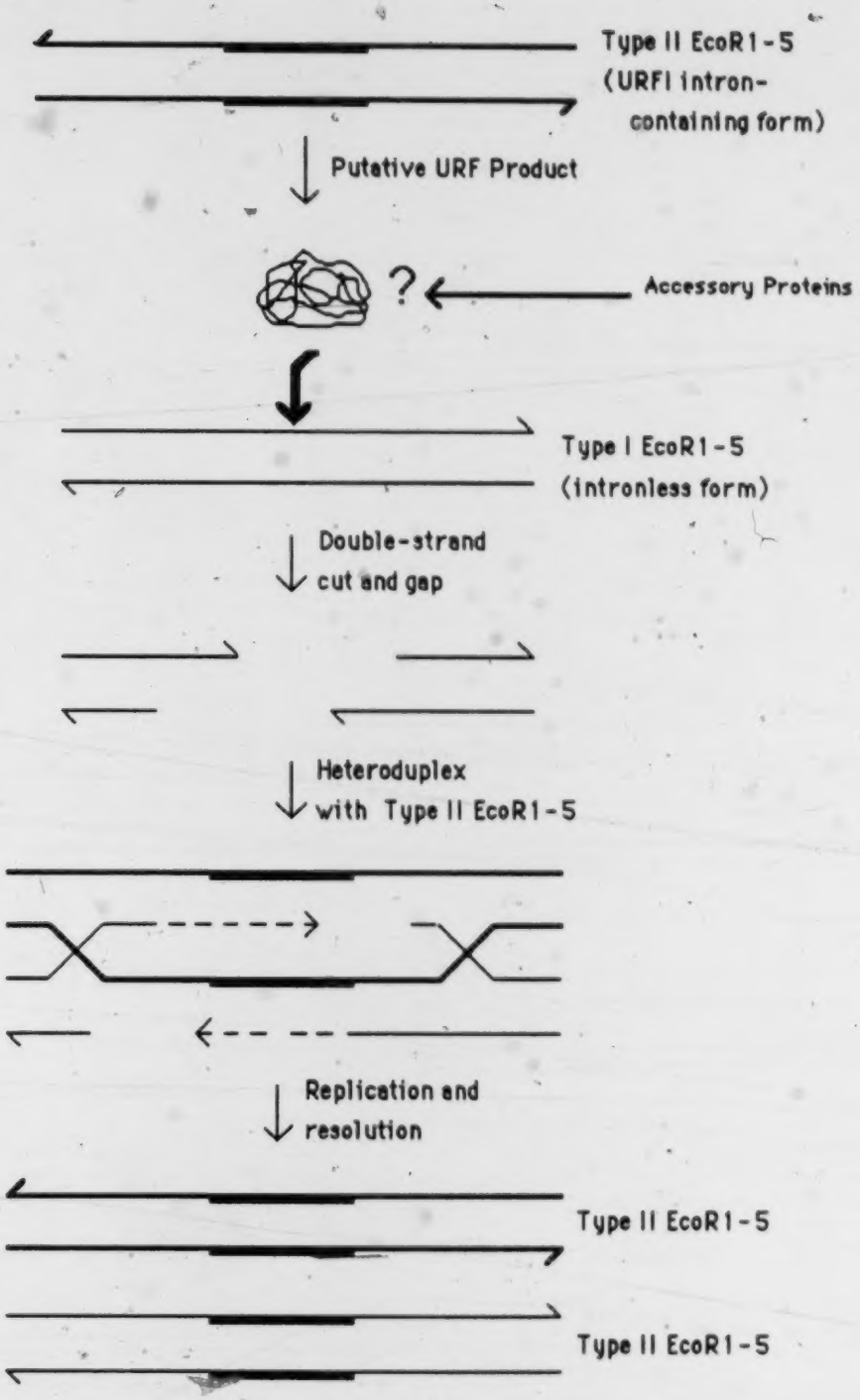

Predicted Splice Site
ATT GGA TCG ATT GCG GAA ACT AAT AGT GCC CCC TTT GAT TTA GCT GAG GCT GAA TCA GAA TTA GTT AGT GGT TTT ATG ACA GAA
ILE GLY SER ILE ALA GLU THR ASN ARG ALA PRO PHE ASP LEU ALA GLU ALA GLU SER GLU LEU VAL SER GLY PHE MET THR GLU

Figure 19

Model for Unidirectional Conversion of the EcoR1-5 Fragment in Heterokaryons Containing Type I and Type II MtDNA

The potential product of the URF1 intronic URF found in type II mtDNA functions of its own or in association with other proteins to create a site specific double-strand cut in the type I mtDNA intronless form of URF1. Double-strand gap repair is then utilized resulting in the observed conversion of type I EcoR1-5 fragments to type II EcoR1-5 fragments. The model is essentially identical to that proposed for the unidirectional conversion of omega in yeast (Zin *et al.*, 1985)

Figure 19


Type II EcoR1-5
(URFI intron-
containing form)
Putative URF Product
?
Accessory Proteins
Type I EcoR1-5
(intronless form)
Double-strand
cut and gap
Heteroduplex
with Type II EcoR1-5
Replication and
resolution
Type II EcoR1-5
Type II EcoR1-5

DISCUSSION

EcoR1-9

The DNA sequence of the type I and type II EcoR1-9 fragments have been determined entirely. The only difference found between the EcoR1-9 type I and II polymorphic forms was the presence of a 78 base pair repeated sequence that is present as three copies in the type I form and as two copies in the type II polymorphic form. A computer search of the EcoR1-9 sequence revealed no tRNAs, and no PstI palindromes or repeats outside the 78 base pair repeat region. Searches were also made for stem-loop structures as well as potential reading frames over 50 amino acids. Although the reading frames were small they appeared to utilize amino acid codons indicative of mitochondrial intron encoded proteins.

The longest potential reading frame spanned the repeat region and it contains a potential start codon at amino acid position 25. In light of what is known about the *var1* mitochondrial gene in yeast, it seemed possible that the ORF spanning the EcoR1-9 repeat region could encode a functional mitochondrial protein even though it contains a variable repeat region. The *var1* gene found in yeast mitochondria encodes a protein of the small mitochondrial ribosome subunit which corresponds functionally to the S5 protein of *Neurosopora crassa* (Burke and RajBhandary, 1985; Butow et al., 1985). The *var1* gene (396+ amino acids) is able to accommodate a GC rich and/or 2 AT rich insertions within its reading frame without affecting the protein's activity (Butow et al., 1985; Zassenhaus and Butow, 1983). It was thought that the 138 amino acid polypeptide that spans the 78 base pair repeat may have some similarities to *var1*. *Var1* is thought to have evolved recently through the recombination of non-transcribed spacer regions (Zassenhaus and Butow, 1983). The conjecture given by the authors is that *var1* evolved through recombination between homologous mitochondrial spacer DNA that carried potential functional domains. The AT rich repeat regions within the 86.9% AT rich*var1* gene are proposed to represent recombination joints (Zassenhaus and Butow, 1983). The repeat region of EcoR1-9 is AT rich (70.5%) and appears to be involved in the recombination process that results in the phenotypic conversion of the EcoR1-9 fragment. If a mechanism similar to that described for *var1* also exists in *Neurospora*, the EcoR1-9 fragment may be involved in a process similar to that Zassenhaus and Butow (1983) describe for the evolution of *var1*. It is possible that the reason the repeat region is always maintained at either two or three copies is

because this open reading frame is expressed. Both the nucleotide sequence of the EcoR1-9 fragment and the amino acid sequence of the potential 138 amino acid polypeptide (which contains the 78 base pair repeat region) were compared to *var1*. No homologies were found by either comparison. None was expected since it was already known that the S5 gene, encoded on EcoRI-1, is the functional equivalent in *Neurospora* . However, the possibility that the EcoR1-9 fragments encode a protein used in the mitochondria can not be ruled out. Many previously unidentified mitochondrial reading frames in *Cephalosporium* (Penalva and Garcia, 1986), Watermelon, (Stern et al., 1986), *Chlamydomonas* (Pratje et al., 1984), and *Neurospora* (Burger and Werner, 1985; Nelson and Macino, 1987) have recently been found to be part of of the Complex I group (NADH dehydrogenase complex) by comparison to those identified in human mitochondria (Chomyn et al., 1983; Chomyn et. al, 1985; Chomyn et al., 1986). However, the Complex I group of proteins are hydrophobic whereas the 138 amino acid protein of EcoR1-9 is relatively hydrophilic and therefore is unlikely to be a member of the complex 1 group.

As stated above, the only sequence difference found between the type I and type II EcoR1-9 fragments was the presence of a 78 base pair repeat present three times in type I and twice in type II. There is some evidence to suggest that such repeats may be responsible for increasing recombination potential. For example, it has been suggested that short sequence homologies are involved in the end-joining step of non-homologous recombination (Roth and Wilson, 1986) and that as few as 25 homologous bases are required to yield recombinant products (Ayares *et al.*, 1986). Repeats have also been implicated in gene amplification of chromosome regions that contain large repeated arrays (Ford and Fried, 1986) and are frequently found associated with recombination events. In addition, nuclear chromosome translocations (Perkins, 1986) and mitochondrial rearrangements (Gross, 1984) have been associated with recombination between repeats. A deletion in the B-globin gene cluster was also found to be the result of recombination between repeats (Henthorn *et al.*, 1986). Fujimoto *et al.* (1985) found inverted R family repeats near the 5' end of the Immunoglobulin J_K gene and suggest that the R repeats may be involved in recombination leading to the rearrangement of the Immunoglobulin genes.

There is also evidence for the involvement of direct repeats in gene conversion events. Chakrabarti and Seidman (1986) found that plasmids carrying a fragmented *SV-40* T-antigen gene (present as direct repeats of segments of the gene with intervening sequences) can reconstitute the gene by conversion when placed in mammalian cells. This process was found to be stimulated by incorporating a double strand break at an appropriate location in the plasmid. Further evidence for the involvement of repeats in recombination and conversion can be found through the examination of multigene families. It is thought that gene conversion between dispersed, repeated, sequences may be responsible for the maintenance of sequence homogeneity in multigene families in *S. pombe* (Amstutz et al., 1985) as well in *S. cerevisiae* (Jackson and Fink, 1985; Jinks-Robertson and Petes, 1986). Toloczyki *et al.* (1986) support the belief that rRNA genes maintain their homogeneity through recombination of spacer regions. They believe that the tandem array of nine 200 bp repeats found in the spacer region of rRNA genes of maize are involved in conversion events that maintain homogeneity of rRNA genes.

The complexity of plant mitochondrial genomes is thought to have arisen from genome rearrangements that involve homologous recombination between repeats (Bailey-Serres *et al.*, 1986). Bailey-Serres *et al.* (1986) report that Maize, *Brassica, Triticum* and *Oenothera* have all undergone homologous recombination between repeats in their mitochondrial genomes. Evidence of recombination between repeats in both mitochondria and chloroplast genomes has been provided by Dron *et al.* (1985) and Howe (1986). Recombination was found to occur at inverted repeats in chloroplast genomes (Howe, 1986) and conversion events are thought to have occurred between two sets of repeats found in *Brassica olerecea's* mitochondrial genome (Dron *et al.*, 1985).

Bernardi (1983) provides evidence that repeats and palindromes are involved in mitochondrial site-specific recombination. He states that direct sequence repeats present in AT rich spacer regions and in GC clusters of the mitochondrial genome are a source of tremendous instability and can function as excision sequences to create petite mutants. Bernardi (1983) suggests that petites may be a product of frequent site-specific recombination events occurring in the wildtype genome that result in excision of mtDNA. In addition, petite genomes could recombine with homologous sequences on the wildtype mitochondrial genome further perpetuating the petite phenotype. This is supported by Perlman and Butow (1987) who report that intra-molecular recombination in yeast mitochondria

leads to rho⁻ and mit⁻ mutants. These mutants are the result of deletions between tandemly repeated sequences that are found throughout the mitochondrial genome (Perlman and Butow, 1987). It has been postulated that there may be a relationship between the creation of some stopper mutants in *Neurospora* and the mechanism of yeast petite formation (Michaelis et al., 1973; Yin et al., 1981). Gross *et al.* (1987) found that recombination between repeated sequences at the mitochondrial tRNAmet gene locus is associated with the formation of a "stopper" mutant.

Gross *et al.* (1984) noted that the *Neurospora* stopper mutants are a dominant phenotype and that during the stopped phase in these mutants, two circular peices of DNA are present which arise as the result of recombination at or near the directly repeated tRNAmet sequence. In the stopper mutant *E35*, both types of mtDNA molecules result from deletions of sequences flanked by short direct repeats. These repeats were part of a larger inverted repeat (de Vries *et al.*, 1986). The stopper mutant *stp-ruv*, contains two circular mtDNA molecules derived by further deletions from an original stopper mutant. The smaller circle produces multimers in high copy number and these are thought to arise from recombination between tandem Pst1 direct repeats and a C rich region adjacent to a sequence containing two 15 bp direct repeats (Gross *et al.*, 1987).

Recombination has also been correlated with replication. Junker *et al.* (1987) found that in *Schizosaccharomyces pombe* intergenic recombination events are non-reciprocal and occur 2-3 times more frequently in meiosis compared to mitosis. Noirot *et al.* (1987) provided direct evidence for plasmid replication stimulating homologous recombination. A plasmid was inserted at varying distances between two direct repeats in the genome of a strain of *Bacillus subtillus* temperature sensitive for recombination. Recombination occurred twenty to four hundred and fifty times more frequently when the plasmids were allowed to replicate (Noirot *et al.*, 1987). Kreuzer and Alberta (1985) have found that some T4 replication origins coincide with phage recombination hot spots. Replication has also been shown to be necessary for homologous and illigitemate excision of *SV40* from the host chromosome (Bullock and Butcham, 1982). Assuming that mitochondria are replicating along with the nuclei in *Neurospora* hyphal tips, one might expect a high frequency of recombination between mitochondrial genomes in heterokaryotic cultures. *Neurospora crassa's* EcoR1-9 fragment contains two direct repeats in the tye II polymorphic form of mtDNA and three copies of the repeat in the type I form. Since repeats appear to be sites of frequent recombination in mitochondria, it seems probable that the conversion of EcoR1-9 is the result of a

recombination event. However, it should be noted that there is no evidence suggesting that this recombination event involves the EcoR1-9 fragment directly. The observed conversion could arise from a crossover that lies between the EcoR1-9 fragment repeat region and the suppressive marker utilized in the study.

From previous reports (Mannella and Lambowitz, 1979; Lemire and Nargang, 1986) and the data provided, the conversion of the EcoR1-9 fragment appears to depend on the phenotype of the parents. In heterokaryons forced between mutant and wildtype strains of *Neurospora crassa* the heterokaryon EcoR1-9 mtDNA fragment is always found to be preferentially converted (70%) to the mtDNA type of the wildtype. Thus, if the wildtype component contained type II mtDNA the heterokaryon EcoR1-9 fragment would be converted preferentially to the type II form (Lemire and Nargang, 1986; this study). If the wildtype component carried type I mtDNA, then the direction of EcoR1-9 conversion is preferentially towards the type I form of DNA (Mannella and Lambowitz, 1979). This phenotypic conversion could result from either a recombinational and/or segregational bias of mtDNA molecules or mitochondria themselves. (Bernardi, 1983; Gross *et al.*, 1987)) The data available point do not suggest a definitive conclusion on this point. Similarly, no conclusions can be reached on the frequency or mechanism of suppressivity of [*mi-3*] mitochondria over wildtype.

EcoR1-5

The EcoR1-5 fragment of *Neurosopora crassa* was previously shown to contain the URF1 gene, which is thought to encode a component of the respiratory chain NADH dehydrogenase complex (Burger and Werner, 1985). The sequence analysis of Burger and Werner was performed on the type II form of EcoR1-5 and also revealed the presence of a large intron within the URF1 gene. This intron was also found to contain a large URF. The sequence analysis performed in the present study revealed that the smaller, type I form of EcoR1-5 arises from the precise excision of the URF containing intron.

The intron of the URF1 gene in the EcoR1-5 fragment of *Neurosopora crassa* appears to follow an inheritance pattern similar to the yeast omega gene (see introduction). In all cases of heterokaryons forced between strains of *Neurospora* containing the type I and type II polymorphic forms of mtDNA, conversion occurs without fail (Lemire and Nargang, 1986; Mannella and Lambowitz, 1978; Mannella and Lambowitz, 1979). This unidirectional conversion of the EcoR1-5 URF1 intron is similar to the conversion of the yeast mitochondrial 21S rRNA gene. The

intronic ORF (omega) of the yeast mitochondrial 21S rRNA gene codes for a site-specific doublestrand endonuclease which is responsible for initiating the conversion of the intron into the intronless strains.(Jaquier and Dujon, 1985; Macreadie *et al.*, 1985; Zinn and Butow, 1985; Colleaux *et al.*, 1986; Kolodkin et al., 1986; Zhu *et al.*, 1987; Zinn and Butow, 1986). Although no sequence homology was found between the omega protein and the URF1 intronic URF, it does not rule out the possibility that the *Neurospora* URF1 intron encodes a functionally similar but unrelated protein that is involved in its own conversion. As discussed below, such a function may be a remnant of a gene carried on an ancient transposable element.

Several lines of evidence suggest that fungal mitochondrial intronic reading frames are the remnants of genes from transposable elements. It was noted by Bonitz *et al.* (1980) that the codon usage of mitochondrial intronic reading frames is characteristically different from the mitochondrial genes themselves. Hudspeth (1982) speculated that this codon bias could be a reflection of colonization of mitochondria by a group of genes not involved in oxidative phosporylation. The observation by Hensgens *et al.* (1983) that some conserved features of mitochondrial intronic ORFs are circularly permuted lead them to speculate that the ORFs may have been inserted via a circular intermediate like that known for the yeast TY element and retroviruses (Boeke *et al.*, 1985; Baltimore, 1985). In addition the location and distribution of group I introns in the mitochondrial genome as well as codon usage by the intronic open reading frames suggest that they arose by insertion into mtDNA genes (Lambowitz *et al.*, 1985). In *Neurospora* and yeast some introns have been shown to be self-splicing in vitro suggesting they may have arisen from an element which could precisely excise itself from the host DNA (Garriga and Lambowitz, 1984; Garriga and Lambowitz, 1986; Banroques *et al.*, 1987).

It has been suggested that some intronic reading frames encode proteins involved in RNA splicing in yeast and *Aspergillus*, as well as *Neurospora* (Davies *et al.*, 1982; Michel *et al.*, 1982; Waring *et al.*, 1982; Michel and Dujon, 1983; Burke *et al.*, 1984; Waring and Davies, 1984; Garriga *et al.*, 1986). It has been proposed that the role of these intronic URFs may be to encode 1) maturase functions 2) proteins involved in the stabilizing of the intron secondary structure important in splicing or 3) components of a mitochondrial-nuclear protein complex involved in RNA splicing (Nevsglyadova, 1984; Lambowitz *et al.*, 1985). Other intronic reading frames may encode functions involving reverse transcriptase

(Akins *et al.*, 1986; Steinhilber and Cummings, 1986). Some intronic maturases have also been suggested to have a regulatory function that evolved from a protein originally involved in transposition (Lambowitz *et al.*, 1985). It is known that the *Neurospora* small mitochondrial ribosome subunit, S5, is an intron encoded protein (Burke and RajBhandary, 1982) which suggests that *Neurospora* may contain other functional intronic proteins.

Two *Neurospora* mitochondrial plasmids (Mauriceville and Varkud) also have properties that suggest they belong to a class of mobile genetic elements that gave rise to mitochondrial introns (Nargang *et al.*, 1984; Lambowitz *et al.*, 1985; Nargang, 1985; Nargang, 1986). These plasmids are closed circular DNAs found in the mitochondria but are not derived from the mtDNA. Sequence analysis of the Mauriceville plasmid revealed conserved regions similar to the conserved sequence elements in group I introns (Nargang *et al.*, 1984). The 710 amino acid ORF encoded in the Mauriceville plasmid utilizes codons distinctive of mitochondrial intron open reading frames. This protein also contains a region of homology to the putative reverse transcriptase of cauliflower mosaic virus and hepititus type B virus which is also homologous to regions of ORFs encoded by group II mitochondrial introns (Nargang *et al.*, 1984; Michel and Lang, 1985). It is also interesting to note that the Mauriceville plasmid transcript's major 3' end and its several major 5' ends can overlap providing short terminal repeats, which is reminiscent of retroviruses and Ty elements (Nargang *et al.*, 1984). Akins *et al.* (1986) found *Neurospora* mutants that appeared to have integrated Varkud and Mauriceville plasmid DNA sequences into the mitochondrial genome via RNA intermediates. It was hypothesized that the plasmid may encode a protein that is responsible for the reverse transcriptase and endonuclease activity required to integrate these plasmids into the mtDNA (Akins *et al.*, 1986). It is believed that recombination was promoted by a site-specific endonuclease, an activity associated with retroviral integration (Akins *et al.*, 1986).

Podospora anserina introns have been implicated in the phenomenon of senescence. The phenomenon of senescence in *Podospora anserina* has been shown to be correlated with the presence of plasmids originating from the amplification of some regions of the mitochondrial chromosome. (Osiewacz and Esser, 1984; Cummings *et al.*, 1985; Belcour and Vierny, 1986) The most common plasmid found in senescing strains is the alpha senDNA (α-senDNA) which is derived from the first intron (class II) of the cytochrome oxidase gene. (Osiewacz and Esser, 1984; Cummings *et al.*, 1985; Belcour and Vierny, 1986) It was

speculated that the URF may encode a splicing enzyme like the yeast maturases (Osiewacz and Esser, 1984). It shows some structural similarity to the Mauriceville plasmid of *Neurospora* in that it is transcribed, its protein has homology to reverse transcriptases and it belongs to the group II intron family (Lang et al., 1985; Nargang, 1985; Akins et al., 1986). It was also shown by Osiewacz and Esser (1984) that this intron contains autonmously replicating sequences like that found in yeast as well as an open reading frame that originates in the upstream exon. The presence of a sequence similar to an autonomously replicating sequence implies the excised α–senDNA may replicate independently and therefore may have been derived from a mobile element. The presence of the autonomous replication sequence in the excised senDNA might cause the plasmid to be suppressive over the mitochondrial genome.

Since the α–senDNA appears to encode a protein involved in the excision of the alpha senDNA fragment (Belcour and Vierny, 1986), other senDNAs found associated with senescing strains in *Podospora* may also encode proteins involved in their own excision and or amplification during the senescence process. The *Podospora* epsilon senDNA (ε-senDNA) plasmid contains a sequence that is homologous to URF1. Its three group I introns, although located at different URF1 sites, have open reading frames located within the intron at the same sites as the intronic URF of *Neurospora's* URF1 gene (Burger and Werner, 1985). Two of the *Podospora* URF1 intronic URFs are homologous to *Neurospora crassa's* URF1 intronic URF (Cummings *et al.*, 1985). The homology between these intronic genes suggests they encode proteins of similar function (Burger and Werner, 1985). The association of these introns with the ε- senDNA suggests that these intronic genes may be involved in the excision of DNA during senescence. Additional factors encoded elsewhere in the nuclear or mitochondrial genome may be required by these ε- senDNAs in order to excise the URF1 introns precisely. The suggestion that intronic URFs are involved in the process of senescence of *Podospora*, and the homology found between *Podospora's* and *Neurospora's* URF1 intronic URF, suggests that the *Neurospora* URF1 intronic URF may encode a protein thats function is involved in the directional conversion of type I mtDNA to the intron containing form of EcoR1-5 (type II).

It has been suggested that excision of senDNA may occur by a number of mechanisms, the first possibility is by recombination. α, β and ε-senDNAs all have been found to contain internal direct repeats that could be involved in site-specific recombination (Cummings *et al.*, 1985). The second possibility is by DNA

splicing by maturase-like enzymes encoded by senDNAs. Finally the senDNAs may be derived from an RNA transcript in young mitochondrial genomes by reverse transcription. It is speculated that a reverse transcriptase may be encoded within the senDNA (Steinhilber and Cummings, 1986). Steinhilber and Cummings (1986) have found evidence of a DNA polymerase activity in "middle aged" cultures of *Podospora* that has characterisitics of a reverse transcriptase. Thus it may be possible that the introns of the sen DNAs were once derived from a transposable element and is no longer able to excise itself properly or once excised, it may become suppressive over the mtDNA due to its ability to replicate independently.

From the observations discussed above, it appears probable that introns could have arisen from mobile genetic elements. The intronic reading frames may have evolved from the proteins encoded in these genetic elements. These genes that were once involved in transposition may have retained their excision and or integration functions, or have evolved into genes with maturase like functions.

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APPENDIX

50x Vogel's Stock Solution

Na3 citrate•$2H_2O$	125g
KH_2PO_4	250g
NH_4NO_3	100g
$MgSO_4$•$7H_20$	10g
$CaCl_2$•$2H_2O$	5g

The first three ingredients were dissolved sequentially in 650 ml of dH_2O. The $MgSO_4$•$7H_20$ and $CaCl_2$•$2H_2O$ were each dissolved in 50 ml then added slowly to the first three dissolved ingredients. The solution was then made up to 1 litre. 5 ml of chloroform was added to the bottle as a preservative.

Vogel's Medium (1 litre)

20	ml	50x Vogel's Stock
15	g	Sucrose (table sugar)
1	ml	Trace Elements (1x)
1	ml	Biotin solution (1x)

Nutritional supplements were added as required. dH_2O was added to make 1 litre.

Vogel's Plates (flasks and slants)

20	ml	50x Vogel's
15	g	Sucrose (table sugar)
2	ml	1x Trace Elements
1	ml	Biotin
880	ml	dH2O
15	g	agar

Supplements were added as required. The solution was made to 1 litre and autoclaved for 20 minutes at 121°C.

20x Westergaard's Stock (1 litre)

20	g	KNO_3
20	g	KH_2PO_4
10	g	$MgSO_4$•$7H_2O$
2	g	NaCl
2.3	g	$CaCl_2$•$2H_2O$

dH_2O was added to make 1 litre and the pH was adjusted to 6.5 with KOH. 10 ml of chloroform was then added as a preservative.

Westergaard's-Sorbose Plates (1 litre)

50	ml	20x Westergaard's Stock
10	g	Sorbose
0.1	g	Glucose
1	ml	Biotin
5	ml	1x Trace Elements
20	g	agar

Supplements were added as required. dH_2O was added to make1 litre and the pH adjusted to 6.5.

Crossing Medium (1x)

50	ml	20x Westergaards
10	g	Table Sugar
5	ml	1x Trace Elements
1	ml	Biotin
20	g	agar

Supplements were added as required. dH_2O was added to1 litre and the pH adjusted to 6.5 using 4N KOH.

4x Trace Elements (1 litre)

50	g	Citric Acid•$1H_2O$
50	g	$ZnSO_4 \cdot 7H_2O$
13	g	$FeNH_4(SO_4)_2 \cdot 12H_2O$
2.5	g	$CuSO_4 \cdot 5H_2O$
0.5	g	$MnSO_4 \cdot 1H_2O$
0.5	g	H_3BO_3
0.5	g	$Na_2MO_4 \cdot 2H_2O$
2	mg	$CoCl_2$

Stored at 4°C.

Biotin Solution (1x)

5 mg Biotin was dissolved in a final volume of 400 ml of 50% ethanol and the solution stored at 4°C.

Nicotinamide

5 g nicotinamide was dissolved in a final volume of 500 ml dH_2O and stored at 4°C. 1 ml of this 10mg/ml solution was used in 1 litre of medium.

Pan

5 g of pantothenate was dissolved in a final volume of 500 ml dH_2O and stored at 4°C. 1 ml of this 10mg/ml solution was used in 1 litre of medium.

Acid-Washed Sand

A 2 litre beaker was filled 3/4 full of Ottawa sand and enough concentrated HCl was added to the beaker to cover the sand. The beaker was covered with a glass plate and left sitting overnight in the fumehood. The following day the acid was poured into an appropriate waste container and the sand transferred to two trays. The trays were placed in the sink and tap water was allowed to continously flow into the sand. The sand was mixed every 5-10 minutes using a gloved hand or an appropriate instrument. The sand was washed with water until the pH was neutral. It was then dryed in the oven and dispensed into jars. The sand was stored at 4°C.

Grinding Buffer
(15% sucrose, 50mM Tris-HCL pH 7.6, 0.25 mM EDTA)

6	g	Tris Base
150	g	Sucrose
1	ml	0.25M EDTA pH8.0
850	ml	dH_2O

The ingredients were dissolved in 600 ml of dH_2O and HCl was added to adjust the pH to 7.6. The volume was made up to 1 litre with dH_2O.

44% Sucrose Solution (1 litre)
(44% Sucrose, 50mM Tris-Cl pH7.6, 0.25mM EDTA)

440	g	ultra-pure Sucrose
6.0	g	Tris Base
1	ml	0.25M EDTA

Add dH_2O to 900 ml.
The pH was adjusted to 7.6 with HCl and the volume brought up to 1 litre.

55% Sucrose Solution (1 litre)
(55% Sucrose, 50mM Tris-Cl pH7.6, 0.25mM EDTA)

550	g	ultra-pure Sucrose
6.0	g	Tris Base
1	ml	0.25M EDTA

Add dH_2O to 900 ml.
The pH was adjusted to 7.6 with HCl and the volume brought up to 1 litre.

60% Sucrose Solution (1 litre)
(60% Sucrose, 50mM Tris-Cl pH7.6, 0.25mM EDTA)

600	g	ultra-pure Sucrose
6.0	g	Tris Base
1	ml	0.25M EDTA pH8.0 Stock

Add dH_2O to 900 ml.
The pH was adjusted to 7.6 with HCl and the volume brought up to 1 litre.

80% Sucrose Buffer
(50mM Tris-Cl pH7.6, 0.10mM EDTA, 80% Sucrose,ultrapure)

10	ml	1M Tris-Cl pH7.5
400	ul	0.25M EDTA
160	g	ultrapure sucrose

Add 50 ml dH_2O
dH_2O was added to a final volume of 200 ml.

0.8M Sucrose Buffer
(0.8M Sucrose, 50mM Tris-Cl pH7.6, 0.25mM EDTA)

274	g	Sucrose
6	g	Tris Base
1	ml	0.25M EDTA

Add 900 ml dH_2O
The pH was adjusted to 7.6 with HCl and the volume brought up to 1 litre.

2.0M Sucrose
(2.0M Sucrose, 50mM Tris-Cl pH7.6, 0.25mM EDTA)

684	g	Sucrose
6	g	Tris Base
1	ml	0.25M EDTA

Add 900 ml dH_2O
The pH was adjusted to 7.6 with HCl and the volume brought up to 1 litre.

10x TBE
(1M Tris-Borate pH 8.3, 20mM EDTA)

Tris Base	121	g
Na2EDTA	6.8	g
Boric Acid	50	g

Add 650 ml dH_2O
The amount of Boric Acid mentioned above was added, then the pH of the solution was adjusted to 8.3 with additional Boric Acid and the volume brought up to 1 litre.

Chloroform/Iso-Amyl Alcohol (24/1,v/v) (1 litre)

960	ml	Chloróform
40	ml	Iso-Amyl Alcohol

The two chemicals were mixed and stored at room temperature.

Loading Dye

18 ml 50% glycerol
2 ml 10x TBE
Add ca. 0.2 mg of bromphenol blue.

RNase A+T1 Stock Solutions
(2mg/ml RNase A, 50% glycerol, H-NET)

20 mg RNase A and 250 ul of RNase T1 solution (sigma) were dissolved in 5 ml H-NET. The solution was boiled for 15 minutes or incubated at 80°C for 30 minutes. 5 ml of glycerol was added and the solution stored at -20°C.

H-NET (10x)
(1M Tris-Cl pH7.8, 1.5M NaCl, 10mM EDTA)

Tris Base	121.14 g
NaC	187.66 g
Na_2EDTA	3.72 g

dH_2O was added to make 1 litre.

H-NET (1x)
(100mM Tris-Cl pH7.8, 150mM NaCl, 1mM EDTA)

1M Tris-Cl Stock	100 ml
5M NaCl Stock	30 ml
0.25M EDTA Stock	4 ml
dH_2O	866 ml

L-Broth

10 g Bacto-tryptone
5 g Yeast Extract
5 g NaCl
1 g Glucose
dH_2O was added to 1 litre. and the pH adjusted to pH 7.4. If plates were to be made 15 g of Bacto-agar was added. The media was autoclaved at 121°C for 20 minutes.

Antibiotics

Antibiotics	Stock (mg/ml)	Final (ug/ml)
Ampicillin[1]	10	50
Streptomycin[1]	100	100
TetracyclineHCl[1,2]	2	20
Chloramphenicol[3]	34	20

1. Dissolve in dH2O, filter sterilize and stored at 4C.
2. Solution is light sensitive. Stored in the dark.
3. Dissolve in 95% ethanol and stored at -20C.

20x D.M.Stock

20 g ammonium sulfate
60 g KH_2PO_4
40 g K_2HPO_4

dH2O was added to 1 litre. 5 ml of chloroform was added for preservative and the solution was stored at 4°C.

D.M. Salts + Thiamine

25 ml 20x D.M. Stock
0.5 ml 10% $MgSO_4$
0.5 ml 10mM Thiamine solution

Supplements were added as required. The solution was made up to a final volume of 500 ml with dH_2O, and dispensed as 10 ml aliquots into 18 X 150 mm testtubes. The tubes were capped with morton closures and autoclaved for 20 minutes at 121°C. !00 ul of sterile 50% glucose was added to the liquid DM media before use

50% Glucose

50g of glucose was added slowly to 50 ml dH_2O. When dissolved it was made to100 ml and dispensed as 1 ml aliquots into eppendorf tubes then autoclaved at 121°C for 20 minutes. The glucose was stored at room temperature.

D.M. PLATES

Flask #1

50 ml 20X D.M. Stock
1 ml 10% MgSO4
1 ml 10 mM Thiamine Solution
448 ml dH_2O

Flask #2

20 g Agar
5 g Glucose
500 ml dH_2O

Supplements were added as required. The flasks were autoclaved separately at 121°C for 20 minutes. The contents of the two flasks were then mixed and poured into sterile petri dishes. The plates were stored at 4°C.

10 mM Thiamine Solution

0.34	g	Thiamine HCl
80	ml	dH_2O

The above were dissolved and dH_2O added to 100 ml. The solution was autoclaved for 20 minutes at 121°C and stored at room temperature.

Transformation Mix #1
(100mM NaCl, 5mM $MgCl_2 \cdot 6H_2O$, 5mM Tris-Cl pH7.6)

NaCl	4 ml of 5M
$MgCl_2 \cdot 6H_2O$	0.2 g
1M Tris-Cl pH7.8	1 ml

Make up to 200 ml with dH_2O
Autoclave or filter sterilize

Transformation Mix #2
(100mM $CaCl_2 \cdot 2H_2O$ (MWT is 147), 250mM KCl, 5mM $MgCl_2 \cdot 6H_2O$, 5mM Tris-Cl pH7.6)

$CaCl_2 \cdot 2H_2O$	2.94	g
KCl	3.8	g
$MgCl_2 \cdot 6H_2O$	0.2	g
1M Tris-Cl pH7.8	1	ml

Make up to 200 ml with dH_2O
Autoclave or filter sterilize.

SOFT AGAR TUBES

10	g	Bacto-tryptone
5	g	Yeast Extract
5	g	NaCl
1	g	Glucose
7	g	Bacto-agar

5 N NaOH was added to a pH of 7.4.
dH_2O was added to 1 litre. The agar was dissolved by heating the media on a hot plate with stirring. The agar was dispensed as 3 ml aliquots into 13 X 100 mm testtubes and capped with morton closures. The tubes were autoclaved at 121°C for 20 minutes then stored at 4°C. Just before use they were autoclaved for 5 minutes at 121°C and placed in a 45°C water-bath.

Alkaline-SDS Solution
(0.2M NaOH, 1% SDS)

200	ul	5N NaOH
500	ul	10% SDS
4.3	ml	dH_2O

Made just before use

Glucose Buffer
(50mM Glucose, 2.5mM Tris-HCL,pH 8.0 at 4°C,10mM EDTA)

.5	ml	1M Tris-Cl pH8.0
0.9	g	Glucose
4	ml	250mM EDTA pH 8.0

dH_2O was added to 100 ml and the buffer stored at 4°C.

TN Buffer (100ml)
(50mM Tris-Cl pH7.8, 0.15M NaCl)

5	ml	1M Tris-Cl pH7.8
3	ml	5M NaCl
92	ml	dH_2O

Stored at 4°C.

25% Sucrose Buffer
(25% Sucrose, 50mM Tris HCl, pH 8.0 at 4°C)

250	g	Sucrose
5	ml	1 M Tris HCl, pH 8.0 at 4°C
500	ml	dH_2O

The above were dissolved and dH_2O was added to a final volume of 1 litre. The buffer was stored at 4°C.

Salt Saturated Isopropanol

100	g	NaCl
250	ml	dH_2O
500	ml	2-propanol (isopropanol)
10	ml	1M Tris-Cl pH7.5

Mixed and stored at 4°C.

10% Sucrose Buffer
(10% Sucrose, 50 mM TrisHCl, pH 8.0 at 4°C)

50	g	Sucrose
25	ml	1 M TrisHCl, pH 8.0 at 4°C
300	ml	dH_2O

The above were dissolved and dH_2O was added to a final volume of 500 ml. The buffer was stored at 4°C.

STE Buffer
(150 mM NaCl, 10 mM TrisHCl, pH 7.8, at 4°C, 1 mM EDTA)

87.66	g	NaCl
100	ml	1 M TrisHCl, pH 7.8 at 4°C
4	ml	0.25 M Na2EDTA, pH 8.0
600	ml	dH_2O

The above were dissolved and dH_2O was added to a final volume of 1 litre. The buffer was stored at 4°C.

10% PEG Solution
(10% PEG, 2.5M NaCl)

100	g	PEG-8000
145	g	NaCl

Add dH_2O to 1 litre.

Protease XI Stock Solution
(3 mg/ml Protease K, 50% glycerol)

30 mg Protease K was dissolved in 5 ml H-NET. The solution was then incubated in 37°C waterbath for 60 minutes. 5 ml glycerol was added and the solution stored at -20°C.

Phenol Extraction Buffer
(0.3 M NaCl, 0.1 M TrisHCl, pH 7.8 at 4°C,1 mM EDTA)

17.5	g	NaCl
100	ml	1M TrisHCl, pH 7.8 at 4°C
4	ml	0.25M Na_2EDTA, pH 8.0
700	ml	dH_2O

The ingredients were dissolved and then dH_2O was added to a final volume of 1 litre. The buffer was stored at 4°C.

NET Buffer
(0.15M NaCl, 0.1mM EDTA, 20mM Tris HCl pH 7.8 at 4°C)

7.5	ml	5M NaCl
0.1	ml	0.25M EDTA pH 8.0
5	ml	1M Tris HCl pH 7.8 at 4°C
150	ml	dH_2O

The above were mixed well and dH_2O was added to a final volume of 250 ml. The buffer was stored at 4°C.

High Salt NET Buffer
(1.0M NaCl, 0.1mM EDTA, 20mM Tris HCl pH 7.8 at 4°C)

50	ml	5M NaCl
0.1	ml	0.25M EDTA 8.0
5	ml	1M Tris HCl pH at 7.8 4°C
150	ml	dH_2O

The above were mixed well and dH_2O was added to a final volume of 250 ml. The buffer was stored at 4°C.

Low Salt Buffer
(0.1 M NaCl, 10mM Tris HCl, pH 7.8 at 4°C, 1mM EDTA)

2	ml	1 M Tris HCl, pH 7.8 at 4°C
4	ml	5 M Nacl
0.8	ml	0.25 M EDTA, pH 8.0
150	ml	dH_2O

The above were mixed well and dH_2O was added to a final volume of 200 ml. The buffer was stored at 4°C.

High Salt Buffer
(1 M NaCl, 10 mM Tris HCl, pH 7.8 at 4°C, 1 mM EDTA)

2	ml	1 M Tris HCl, pH 7.8 at 4°C
40	ml	5M NaCl
0.8	ml	0.25 M EDTA, pH 8.0
150	ml	dH_2O

The above were mixed well and dH_2O was added to a final volume of 200 ml. The buffer was stored at 4°C.

Sequencing Mixes
Regular Mixes
(Note: Stocks are stored at -70°C; mixes are stored at -20°C.)

A mix:

40 ul	0.5 mM dCTP
40 ul	0.5 mM dGTP
40 ul	0.5 mM dTTP
40 ul	10X Pol Buffer
140 ul	dH_2O
1 ul	10 mM ddATP

Note: for "long" A mix use 0.5 ul 10 mM ddATP; for "extra long" A mix use 0.25 ul 10 mM ddATP.

C mix:

4 ul	0.5 mM dCTP
40 ul	0.5 mM dGTP
40 ul	0.5 mM dTTP
40 ul	10X Pol Buffer
120 ul	dH_2O
2 ul	10 mM ddCTP

Note: for "long" C mix use 1.0 ul 10 mM ddCTP.

G mix:

40 ul	0.5 mM dCTP
4 ul	0.5 mM dGTP
40 ul	0.5 mM dTTP
40 ul	10X Pol Buffer
110 ul	dH_2O
4 ul	10 mM ddGTP

Note: for "long" G mix use 1.5 ul 10 mM ddGTP.

T mix:

40 ul	0.5 mM dCTP
40 ul	0.5 mM dGTP
4 ul	0.5 mM dTTP
40 ul	10X Pol Buffer
100 ul	dH_2O
8 ul	10 mM ddTTP

Note: for "long" T mix use 2.5 ul 10 mM dTTP; for extra long T mix use 1 ul 10 mM ddTTP.

Chase:

20 ul	10 mM dATP
20 ul	10 mM dCTP
20 ul	10 mM dGTP
20 ul	10 mM dTTP
120 ul	dH_2O

dITP Mixes

(Note: Stocks are stored at -70°C; mixes are stored at -20°C.)

A mix:

20 ul	0.5 mM dCTP
100 ul	0.5 mM dITP
20 ul	0.5 mMdTTP
20 ul	10X Pol Buffer
40 ul	dH_2O
0.25 ul	10 mM ddATP

C mix:

2 ul	0.5 mM dCTP
100 ul	0.5 mM dITP
20 ul	0.5 mM dTTP
20 ul	10X Pol Buffer
60 ul	dH_2O
1.5 ul	10 mM ddCTP

G mix:

20 ul	0.5 mM dCTP
3 ul	0.5 mM dITP
20 ul	0.5 mM dTTP
20 ul	10X Pol Buffer
140 ul	dH_2O
1.5 ul	1 mM ddGTP

T mix:

20 ul	0.5 mM dCTP
100 ul	0.5 mM dITP
2 ul	0.5 mM dTTP
20 ul	10X Pol Buffer
60 ul	dH_2O
4 ul	10 mM ddTTP

Chase:

20 ul	10 mM dATP
20 ul	10 mM dCTP
20 ul	10 mM dGTP
20 ul	10 mM dTTP
120 ul	dH_2O

Deaza-G mixes (Barr et al. 1986)

Deaza-G mixes were made the same as normal mixes except 10mM 7-Deaza-2'-deoxyguanosine-5'-triphosphate was used instead of 10mM dGTP.

Formamide Stop Dye (for sequencing)

9.5 ml	deioniozed formamide (prepared by adding mixed bed resin(1 gram/10 ml formamide) and letting solution stand 30 minutes then filtering
400 ul	0.25 M EDTA stock
10mg	Bromphenol Blue
10mg	Xylene cyanole

Mixed and stored at -20°C.

Carrier DNA

2 mg of herring sperm DNA dissolved in 1 ml dH_2O then sonicated for 1 hr.
Stored at 4°C.

100 X Denhardt's

2% (w/v) ficoll
2% (w/v) polyvinyl pyrrolidone
2% (w/v) bovine serum albumin
Stored at 4°C

20 X SSPE
(3.6M NaCl, 0.2 M Naphosphate, pH 8.3, 0.02 M EDTA)

318.4	g	NaCl
25.2	g	Na_2SO3
80	ml	0.25 M EDTA pH8.0 Stock

Mix and stored in freezer.

Prehybridization Solution

for 10 mls:

1 ml	50 X Denhardt's
2.5 ml	20 X SSPE
6.3 ml	dH_2O
100 ul	10 mg/ml carrier DNA

Stored at -20°C. Use 4 mls for 100 cm^2 membrane

Hybridization Solution

Place 800 ul of prehybridization solution in an eppendorf and add ^{32}P probe (10^7 - 10^8 cpm/ug DNA). Denature in a boiling water bath for 2 minutes.

Solution O
(1.25 M TrisHCl, pH 7.8 at 4°C, 0.125 M $MgCl_2$, pH 8.0)

7.57 g Tris Base
1.27 g $MgCl_2$

Dissolve Tris base in 40 ml dH_20 and pH to 7.8 with HCl. Add $MgCl_2$ and bring volume up to 50 ml. Stored in 1 ml aliquots at 4°C

Solution A

1 ml solution O
18 ul ß-mercaptoethanol
5 ul (0.1M) dATP
5 ul (0.1M) dTTP
5 ul (0.1M) dGTP

Stored at -20°C.

Solution B

2 M Hepes titrated to pH 6.6 with 4 M NaOH. Stored at 4°C.

Solution C

Hexadeoxyribonucleotides (BRL) suspended in TE at 90 OD units. Stored at -20°C.

OLB

Mix A:B:C in a ratio of 100:250:150. Stored at -20°C for up to 3 months.

Stop Buffer

2 mM NCl, 20 mM Tris-HCl, pH 7.8 at 4°C, 2 mM EDTA, 0.25% SDS, 1 uM dCTP. Stored at -20°C.

18% Sodium Sulfite

220 g Na_2SO_3

Make up to 1 litre with dH2O.

Photoflow

10 ml Photflow

Make up to 1 litre with dH_2O.